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Nuevas estrategias para la prevención y control de la peste porcina africana

New Strategies for fhe Prevention and Control of African Swine Fever

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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Nuevas estrategias para la prevención y control de la Peste porcina africana

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New strategies for the prevention and control of African swine fever

Memoria de Tesis doctoral

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CERTIFICAN

Que Dª Lina Mur Gil, licenciada en Veterinaria, ha realizado bajo su dirección el trabajo titulado "Nuevas estrategias para la prevención y control de la Peste porcina africana", considerando que reúne los requisitos necesarios para ser presentado como Tesis Doctoral.

En Madrid, a 14 de Julio de 2014

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La verdadera grandeza de la ciencia
acaba valorándose por su utilidad

D. Gregorio Marañón (1887-1960)

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LISTADO DE ABREVIATURAS

- ADS: Asociación de Defensa Sanitaria
- CEE: Comunidad Económica Europea
- Dpi: días post-infección
- EFSA: European Food Safety Authority
- ELISA: Enzyme-linked immunosorbent assay/ Ensayo de inmunoadsorción ligado a enzimas
- EU: European Union
- EUROSTAT: Statistical Office of the European Communities/ Oficina Estadística de las Comunidades Europeas
- Hpi: horas post infección
- HRP: High Risk Period/ Periodo de alto riesgo
- IB: Immunoblotting
- IPT: Immuno peroxidase technique/ Técnica de la Inmunoperoxidasa
- nm: nanómetro
- OE: *Ornithodoros erraticus*
- OIE: Organización Mundial de Sanidad Animal
- OMC: Organización Mundial de Comercio
- pi: post-infección
- PCR: Polymerase chain reaction/Reacción en cadena de la polimerasa
- PCV-2: Circovirus porcino tipo 2
- PPA: Peste porcina africana
- PPC: Peste porcina clásica
- PRRS: Síndrome respiratorio y reproductor porcino
- RAT: Riesgos Asociados al Transporte
- SCI: Science Citation Index
- UE: Unión Europea
- TRACES: Trade Control and Expert System/ Sistema informático veterinario integrado
- vPPA: virus de la Peste porcina africana

1. RESUMEN

La Peste porcina africana (PPA) es una de las enfermedades más complejas y de mayor importancia de cuantas afectan al ganado porcino. Es una enfermedad de declaración obligatoria, cuya presencia conlleva importantes repercusiones socio-económicas para los países que la padecen. Actualmente la PPA está presente de forma endémica en un gran número de países del continente africano, lo que genera importantes pérdidas económicas y supone un peligro constante por la posible difusión a países libres de otros continentes. Una clara demostración de este hecho son las dos últimas salidas del virus de la PPA (vPPA) a Europa (1960 y 2007), que a día de hoy continúan ocasionando problemas tanto en Cerdeña (afectada desde 1978) como en el Este de Europa (desde 2007). Precisamente, el escaso éxito en el control de la PPA en el Este de Europa, llegando incluso a alcanzar algunos países de la Unión Europea (UE) (Lituania, Polonia y Letonia), ha elevado el nivel de alarma frente a este virus, hasta el momento relativamente olvidado.

Esta tesis doctoral titulada "**Desarrollo de nuevas estrategias para la prevención y el control de la Peste porcina africana**" tiene como objetivo desarrollar y proporcionar nuevas metodologías y herramientas que permitan mejorar tanto los sistemas de prevención y vigilancia, como los programas de control frente a la PPA en Europa. El trabajo aquí desarrollado ha sido divulgado en nueve artículos científicos publicados en revistas del SCI (Science Citation Index), en los que se incluyen diversas metodologías de análisis de riesgo, estudio de factores de riesgo y modelos de control en zonas endémicas, así como el desarrollo de nuevos métodos diagnósticos para la vigilancia de la enfermedad.

En el primer objetivo se realizó una evaluación epidemiológica de la PPA, describiendo los distintos escenarios existentes en la actualidad, analizando en detalle el avance de la enfermedad en el Este de Europa durante los primeros cincos años. A continuación, se identificaron los factores de riesgo que han podido propiciar su rápida difusión y dificultan el control en el Este de Europa. Entre ellos destacan: la ausencia de un programa de control coordinada y financiado a nivel estatal, la abundante presencia de cerdos de traspatio y otras producciones de baja bioseguridad, el comercio ilegal de cerdos y productos y el frecuente uso de desperdicios para la alimentación animal. La mayoría de estos factores son del tipo socio-cultural, lo que dificulta en gran medida su cambio y favorece la endemicidad de la enfermedad en la zona. Estos estudios nos mostraron la

evolución epidemiológica de la PPA y el peligro asociado a su actual distribución de la PPA, especialmente para algunos países de la UE.

En el segundo objetivo se desarrollaron dos análisis de riesgo que permitieron cuantificar la distribución espacial y magnitud del riesgo de entrada del vPPA a través de dos de las vías de entrada más importantes. Se empleó un modelo riesgo cuantitativo para el análisis del riesgo derivado del comercio legal de cerdos, que estimó un riesgo bajo de introducción del vPPA en la UE asociado actualmente a esta vía ($5.22*10^{-3}$), que corresponde aproximadamente con un brote cada 192 años. Por lo que esta vía no supone una prioridad actual en los programas de vigilancia y control de la UE. Por el contrario, el riesgo asociado a transportes se evaluó mediante un modelo de riesgo semi-cuantitativo, en el que Polonia (4,13/5) y Lituania (3,8/5) concentraron los valores más elevados de riesgo. En esta vía, el riesgo asociado a camiones de regreso concentró los valores más altos de riesgo (65% del riesgo por esta vía), seguido de los desperdicios de barcos internacionales y finalmente de aviones.

Además, se desarrolló una herramienta modular que permitió la integración y visualización conjunta de los análisis de riesgo realizados para todas las vías de introducción de PPA en la UE. Esta herramienta incluye tanto las dos vías aquí analizadas, como otras tres vías de entrada desarrolladas en un proyecto europeo, en cuyos análisis también colaboramos. Esta herramienta permite la rápida visualización y análisis detallado del riesgo para cada país y para cada vía potencial de entrada. En dichos resultados se observa como un 48% de los países de la UE presentó un valor de riesgo relativo elevado para alguna de las vías analizadas, existiendo cuatro países con el valor de máximo riesgo para al menos una de ellas. Se observaron además, importantes diferencias entre las rutas analizadas, ya que mientras en algunas el riesgo estaba concentrado en pocos países (fómites asociados al transporte), otras afectaban a más de 4 y 5 países (importaciones legales de cerdos, movimiento de jabalíes o importaciones ilegales). Esta herramienta y los resultados incorporados en ella podrán ser empleados por los distintos países miembros de la UE para la adaptación y actualización de los programas de vigilancia y prevención en base al riesgo existente. A su vez, la herramienta modular aquí desarrollada se ha publicado como material suplementario en una revista de libre acceso, por lo que se encuentra disponible de forma libre, incluyendo una descripción transparente de los parámetros, lo que permite su uso para la estimación de otros riesgos en otras zonas, regiones o periodos de tiempo.

El tercer objetivo se centra en el estudio de los factores de riesgo que dificultan el control de la enfermedad en territorios endémicos. En el primer estudio, tomando Cerdeña como escenario, se realizó inicialmente una revisión de la evolución de la PPA durante los 35 años de presencia en la isla, identificando los factores de riesgo que han posibilitado su permanencia a través de los años. Destacan entre ellos la abundancia de granjas de baja o nula bioseguridad en Cerdeña, la presencia de cerdos en libertad no identificados (ilegales), así como la alta densidad de jabalí en la isla. Se desarrolló una metodología basada en la combinación de modelos de difusión y modelos estadísticos para la evaluación de los factores de riesgo para PPA presentes en zonas endémicas, tomando como modelo Cerdeña. El empleo de un modelo de difusión para simular la enfermedad en el cerdo doméstico reveló un tamaño pequeño de los brotes (1,7 granjas infectadas de media), donde la difusión local es la principal vía de transmisión. Estos resultados demostraron la relación directa entre la bioseguridad y control de las explotaciones, con la potencial difusión de la enfermedad en Cerdeña. Por el contrario, el desarrollo de cuatro modelos de regresión logística demostró la gran importancia de los cerdos ilegales y los jabalíes en la ocurrencia de PPA, siendo en general más importante la influencia de los ilegales. Sin embargo, cuando la densidad de jabalí fue muy elevada, este factor presentó una influencia aún mayor que la presencia de ilegales. En base a estos resultados se construyeron unos mapas de riesgo de difusión y ocurrencia de PPA en Cerdeña que permitirán focalizar las actividades de control y dirigirlas a los factores de riesgo más importantes presentes en cada zona. Se espera que la adaptación de medidas de control a los distintos factores identificados, resulte en una mejora de la eficacia de los programas y una reducción de la prevalencia de PPA en Cerdeña.

En paralelo, se realizó un estudio retrospectivo en una región de España para establecer la importancia de los jabalíes en el ciclo epidemiológico y su capacidad para mantener la infección de PPA en el ambiente. Para ello, se evaluaron 158 muestras recogidas procedentes de una de España endémica de PPA, donde la erradicación de PPA fue más difícil por la presencia de explotaciones de cerdo extensivas, garrapatas y jabalíes. Los resultados de este estudio revelaron la incapacidad del jabalí para mantener la infección en ausencia de otras fuentes de contaminación externa. Las metodologías empleadas en estos trabajos podrán ser aplicadas a otras regiones, con el fin de identificar y evaluar la importancia de los factores de riesgo presentes en las zonas afectadas, cuyo control es fundamental y necesario para lograr la erradicación de la enfermedad.

En cuarto y último objetivo, con el fin de facilitar la vigilancia de PPA, se adaptaron técnicas de diagnóstico serológico para la detección de anticuerpos frente a PPA en fluido oral. Los resultados de este estudio demostraron la presencia de anticuerpos frente al vPPA en el fluido oral de los animales infectados, y la capacidad de detectarlos mediante el ajuste de dos técnicas serológicas (ELISA e IPT) ya existentes. Estos resultados abren la puerta a la futura aplicación de esta muestra en la vigilancia serológica de PPA para la detección de animales portadores y supervivientes. Por el contrario, su uso en casos de PPA agudos con presencia de elevada fiebre y mortalidad repentina es más difícil, ya que los animales enfermos pierden el interés por la cuerda y esto imposibilita la obtención de muestra. El empleo de esta muestra presenta bastantes ventajas en relación a la utilización de otras muestras biológicas, evitando el sangrado de los animales, mejorando el bienestar y manejo de los mismos. A su vez, al tratarse de un método no invasivo, su uso permitiría la vigilancia de poblaciones silvestres sin necesidad de capturarlas, mediante el uso de sustancias atrayentes, de una forma rápida, sencilla y fiable.

Los resultados y metodologías desarrolladas en la presente tesis son novedosos con una gran aplicación para el desarrollo y actualización de los programas de vigilancia en la UE, ya que presentan una caracterización específica del riesgo para cada uno de ellos. A su vez, la identificación y priorización de factores de riesgo en territorios endémicos es una herramienta de gran utilidad para el diseño de estrategias de control en territorios afectados. Estos avances, sumados a la potencial aplicación de las nuevas muestras diagnósticas mediante el uso del fluido oral, se espera resulten en una mejora de la prevención de la entrada de PPA en países libres, y un control más efectivo de la enfermedad en los territorios afectados.

2. ABSTRACT

African swine fever (ASF) is one of the most complex and important diseases of swine. As it is a mandatory notifiable disease, its presence implies important socio-economic consequences in affected countries. Nowadays, ASF is endemic in a lot of countries within the African continent, posing a permanent risk of spread to free countries in other continents. This fact was clearly demonstrated by the last two escapes of ASF virus (ASFV) into Europe (1960 and 2007), that still nowadays are causing problems both in Sardinia (affected since 1978) and East Europe (affected in 2007). The lack of control and continuous spread in East Europe, even reaching European Union (EU) countries, has raised the awareness on this disease, partially forgot during these last decades.

This PhD thesis entitled "**Development of new strategies for the prevention and control of African swine fever"** has the goal of developing and providing new methodologies and tools for improving prevention and surveillance systems, as well as control programs against ASF in Europe. The work herein developed is summarized in nine scientific articles published in SCI (Science Citation Index) journals. These publications contain risk assessment methodologies, risk factors studies, models for control in endemic areas, as well as new methodologies for ASF surveillance.

The first objective includes the evaluation of the epidemiological situation of ASF, including the description of the ASF epidemiological scenarios currently present, as well as the detailed evolution and spread of the disease in Eastern Europe during the first five years of the epidemic. In addition, the risk factors present in East Europe that could be involved in the rapid spread of ASF and difficult its control, were identified. These risk factors include the absence of a coordinated and funded national control program in the Russian Federation, the abundant presence of back-yard pigs and other production systems with low biosecurity standards, and the frequent practice of swill feeding in the area. Most of these factors are socio-cultural, so their change would result very difficult, and consequently, the probability of ASF become endemic in the area is very high. These studies updated the information available about ASF epidemiology, enhancing the risk associated to the current distribution of ASF, specifically for some EU countries.

Consequently, the second objective consisted on the development of risk assessment studies for estimating the location and magnitude of the risk for ASFV introduction into EU through two of the most important routes of introduction. Specifically, the risk derived from the imports of live pigs during the high risk period was analyzed by a quantitative stochastic model. This model revealed a low probability of ASFV introduction by this pathway into the EU ($5.22*10^{-3}$, which corresponds approximately with one outbreak every 192 years). Therefore, this route is not a current priority for the surveillance and control programs of the EU. In contrast, the risk associated to transport routes was estimated by the use of a semi-quantitative model, revealing that this risk was mainly concentrated in Poland (4.13/5) and Lithuania (3.8/5). The returning trucks was the sub-pathway at higher risk (65% of the total risk), followed by the waste form international boats and, ultimately, by the waste from international planes.

In addition, a modular tool was developed for the integration and joint visualization of all the risk assessments studied, including not only the two pathways analyzed in this thesis, but also three other pathways evaluated within a European project, in which assessments we also collaborated. This tool allows the global visualization and analysis of the results, revealing that 48% of EU countries presented a relative high risk for some of the routes analyzed. Important differences were observed among the different routes; while in some of them the risk was concentrated in few countries (transport associated fomites), others affected to 4 or 5 countries (legal imports of pigs, wild boar movement or illegal imports). This tool and the results provided in it could be used by EU member states for the adaptation and update of their surveillance and prevention programs based on the current existing risk. This modular tool developed herein has been published as supplementary material in an open access journal, so it is freely available, and it can be used for estimating ASF risk into other regions or periods of time.

The third objective is focused on the study of risk factors that difficult ASF control in endemic situations. The first study, located in Sardinia, evaluated ASF evolution during the 35 years of presence in the island, and identified the critical points that could have favored the persistence of the disease in the island. The abundant presence of small farms with low or null biosecurity measures, the presence of free non-controlled pigs (illegal) and the high density of wild boar in the island, were the most important identified factors. A new methodology based on the combination of spread and statistics models was developed for the evaluation of risk factors' influence in endemic areas, as Sardinia. Firstly, we employed a disease spread model to simulate ASF spread within the domestic compartment, that reveals a general low spread of the disease

(1.7 outbreaks on average), mainly caused by local spread. The comparison of these results with the ASF outbreaks occurred in Sardinia from 2007 to 21013, revealed a direct correlation between the biosecurity and control measures of the holdings and the potential spread of ASF in Sardinia. In contrast, the development of four logistic regression models revealed that both, the presence of illegal pigs and the density of wild boars are significant correlated with ASF occurrence. In general, illegal pigs have a higher influence; however, when very high densities of wild boar are present, this factor is more important. All these results were employed for creating risk maps for ASF spread and occurrence that would allow identifying the high risk areas for each factor, and consequently the priority areas to act. The adaptation of control measures to the specific factors identified in each area would result in an improvement of the efficacy of the programs, and a potential reduction of ASF prevalence in the island.

In parallel, a retrospective study was performed in a specific region of Spain for evaluating the importance of wild boar in ASF epidemiology, and its capability for maintaining ASF in the environment. For that purpose, 158 wild boar samples collected from one of the regions of Spain previously endemic for ASF, where ASF was most difficult to eradicate due to the presence of extensive pig production, ticks and wild boar, were evaluated. All of the samples were negative, revealing the inability of wild boar for maintaining ASF without the implication of other external sources of contamination. The methodologies developed and employed in these studies could be used in other regions for identifying and evaluating the role of risk factors present in the area. The adaptation of control and eradication programs to these risk factors is essential for the final eradication of the disease.

The fourth and last objective aims to facilitate ASF surveillance by the adaptation of serological techniques for the detection of antibodies against ASF in oral fluid. This study demonstrated for the first time, the presence of ASF antibodies in oral fluid of infected animals and the possibility of detecting them by the adjustment of two serological techniques (ELISA and IPT) previously available for serum samples. These results suggest the possibility of using this sample for ASF serological surveillance for the detection of survivor and carrier animals. Conversely, the potential use of oral fluid for the diagnosis of ASF acute forms is unprovable, as with the early appearance of high fever and mortality, diseased animals don’t react to the rope and it is almost impossible to obtain the sample. However, for serological surveillance, this sample presents several advantages comparing with serum samples. Firstly, it does not require bleeding animals, benefits welfare and animal management, and importantly, it is a non-invasive

method that could be used for surveillance of wild populations by the use of some inviting substances in a simple and rapid way.

Results and methodologies developed in this thesis are novel and important with a direct application for the development and update of surveillance programs in the EU, as they provide a risk categorization for each of the countries. The identification and evaluation of risk factors in endemic territories is a useful tool for the design of control strategies in affected territories. These developments, together with the potential application of new diagnostic techniques for the diagnosis of oral fluid samples, are expected to result in an improvement of prevention of ASF entrance into EU, and a more effective control in ASF affected territories.

Il terribile spettro della peste suina

di Antonio Bassu

3. INTRODUCCIÓN

Fuente: La nuova Sardegna, 19 Agosto 1980.

3. INTRODUCCIÓN

La Peste porcina africana (PPA) es una de las siete enfermedades de los suidos incluida en la Lista de enfermedades declaración obligatoria de la Organización Mundial de Sanidad (OIE). Como su nombre indica, fue descubierta en África, donde se encuentra ampliamente presente en la actualidad. Sin embargo, en diversas ocasiones se ha difundido más allá de las fronteras del continente africano y, en dos de las tres ocasiones que ha alcanzado Europa, ha ocasionado importantísimos problemas tanto sanitarios como económicos.

La primera salida del continente africano ocurrió en 1957, infectando Portugal pero con limitada difusión y repercusiones. La segunda introducción en Europa ocurrió en 1960, siendo esta mucho más importante, ya que tras ella la PPA se difundió con rapidez por la Península Ibérica (España y Portugal), donde estuvo presente más de 30 años ocasionando además numerosos brotes en otros países y continentes. De nuevo, en 2007, cuando Europa parecía haberse olvidado de este virus (a excepción de Cerdeña), la PPA alcanzó el Este de Europa, esta vez a través de Georgia. Desde entonces la enfermedad ha ido avanzando poco a poco sin freno, llegando incluso a afectar a países de la Unión Europea (UE) (Lituania, Polonia, Letonia).

En este contexto de preocupación generalizada por el control de la PPA para frenar su avance, se desarrolla esta tesis. Con el objetivo de desarrollar nuevas herramientas y estrategias que ayuden tanto a prevenir la entrada de la enfermedad en los países libres, como para asistir al diseño de planes de control específicos adaptados a cada país con el objetivo de controlar y erradicar la infección en los territorios afectados.

3.1. PRINCIPALES CARACTERÍSTICAS DEL VIRUS DE LA PESTE PORCINA AFRICANA

3.1.1. El virus

La PPA está producida por el virus de la PPA (vPPA), único miembro actual de la familia *Asfarviridae* (Dixon *et al.*, 2005). Es un virus de gran tamaño, cuya partícula viral presenta una morfología icosaédrica con presencia de cuatro capas concéntricas (el núcleo central, la envuelta del núcleo, la membrana interna y la cápside icosaédrica). A ellas se une una membrana hexagonal externa adquirida durante el proceso de gemación, confiriéndole un tamaño aproximado de 200 nm de diámetro (Salas y Andrés, 2013).

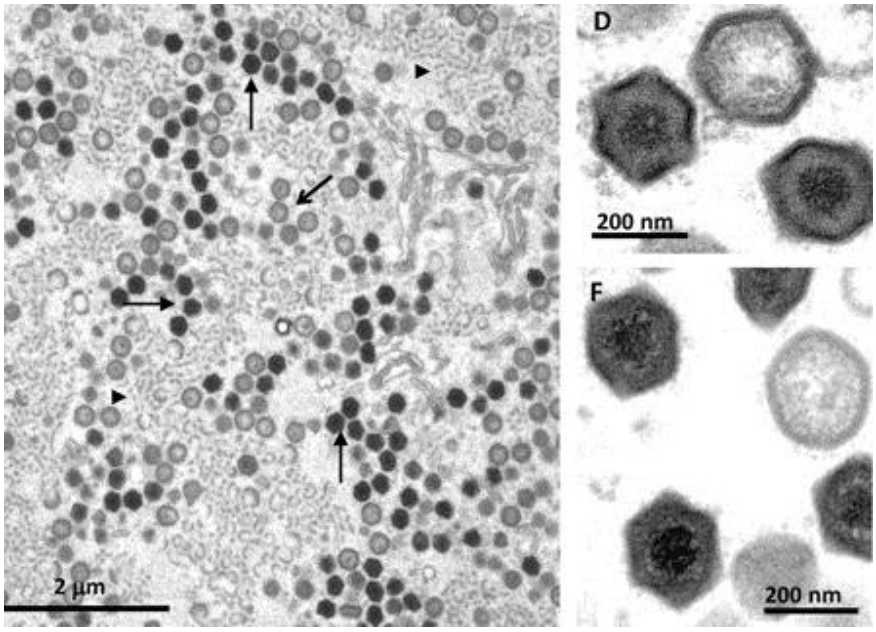

Figura 1: Imagen del vPPA en microscopia electrónica (Fuente:(Rodríguez *et al.*, 2009))

Su material genético se encuentra presente en forma de DNA de doble cadena, de gran extensión entre 170 y 193 kpb según el aislado viral. Se diferencia una región central hipervariable flanqueada por dos extremos genéticamente más conservados unidos covalentemente (Dixon *et al.*, 2013). Contiene entre 150 y 167 marcos de lectura que codifican la expresión de más de 150 proteínas de infección. De todas ellas, 54 proteínas han sido descritas como componentes estructurales que intervienen en el ensamblaje y formación de los viriones. No obstante, a día de hoy tan sólo 19 genes codificantes de estas proteínas han sido identificados, conociéndose la funcionalidad de tan sólo 9 de ellas (Salas y Andrés, 2013).

Estos datos nos dan una idea de la complejidad del vPPA, sobre todo si lo comparamos con otros virus causantes de enfermedades animales con formas clínicas similares. Por ejemplo, el virus de la Peste porcina clásica (PPC) de la familia *Flaviviridae* está formado por una partícula vírica de unos 40-60 nm de diámetro. Su material genético en forma de ARN simple de polaridad positiva, abarca unos 12 kpb, y codifica tan solo para cuatro proteínas estructurales y otras 7 proteínas no estructurales de infección (Moennig, 2000).

En el caso de PPA, alrededor de 50 de las proteínas virales son inmunogénicas, siendo algunas de ellas capaces de provocar una respuesta inmune de gran intensidad. Destacan por su papel antigénico la proteína p72 de la cápside viral y las proteínas p54, p30 y p12 de las membranas, que constituyen la base de la mayoría de las técnicas serológicas actualmente

empleadas. Si bien existen otras proteínas del virus (pK205R y pB602L), que también muestras interesantes propiedades como diana serológicas (Gallardo *et al.*, 2009b, Gutierrez-Castaneda *et al.*, 2008).

La infección del vPPA induce la aparición en pequeña proporción de anticuerpos parcialmente neutralizantes frente a algunas proteínas virales (Gomez-Puertas *et al.*, 1996, Zsak *et al.*, 1993). Sin embargo, estos anticuerpos no son suficientes para neutralizar por completo la infección viral (Neilan *et al.*, 2004). A pesar de la falta de neutralización, la inmunidad humoral juega un papel importante en la protección frente al vPPA, permitiendo retrasar la aparición de la enfermedad, e incluso proteger frente a infecciones posteriores, tal como demostró un experimento mediante transferencia pasiva de anticuerpos (Onisk *et al.*, 1994).

La ausencia de anticuerpos completamente neutralizantes ha sido una de las principales dificultades para la obtención de una vacuna eficaz.

No obstante, no sólo la inmunidad humoral es importante en la protección frente al vPPA. Estudios anteriores demostraron el importante papel de la inmunidad de tipo de celular en la protección frente al virus, especialmente de los linfocitos CD8 (Oura *et al.*, 2005) y de las células natural killer (Leitão *et al.*, 2001). Estos descubrimientos abrieron paso a nuevas líneas de investigación en búsqueda de una vacuna eficaz, así como del estudio de efectos negativos del sistema inmune celular sobre la patología de la PPA (Takamatsu *et al.*, 2013).

El vPPA infecta principalmente a macrófagos y monocitos del sistema mononuclear fagocitario, lo que determina en gran medida la patogenia ocasionada por el vPPA y su gran capacidad para evadir los mecanismos del sistema inmune del hospedador (Wardley y Wilkinson, 1978, Malmquist y Hay, 1960).

El vPPA también es capaz de infectar, aunque en menor medida, células endoteliales (Carrasco *et al.*, 1996), hepatocitos (Gómez-Villamandos *et al.*, 1995b), células del epitelio tubular renal (Gomez-Villamandos *et al.*, 1995) y neutrófilos. Sin embargo, hasta la fecha no se ha demostrado la infección de linfocitos T ni B (Minguez *et al.*, 1988, Carrasco *et al.*, 1996).

La infección por el VPPA provoca apoptosis en linfocitos y linfopenia asociada, lo cual influye en la capacidad de respuesta inmune frente al virus (Sanchez-Vizcaino *et al.*, 1981, Gómez-Villamandos *et al.*, 1995a, Oura *et al.*, 1998b).

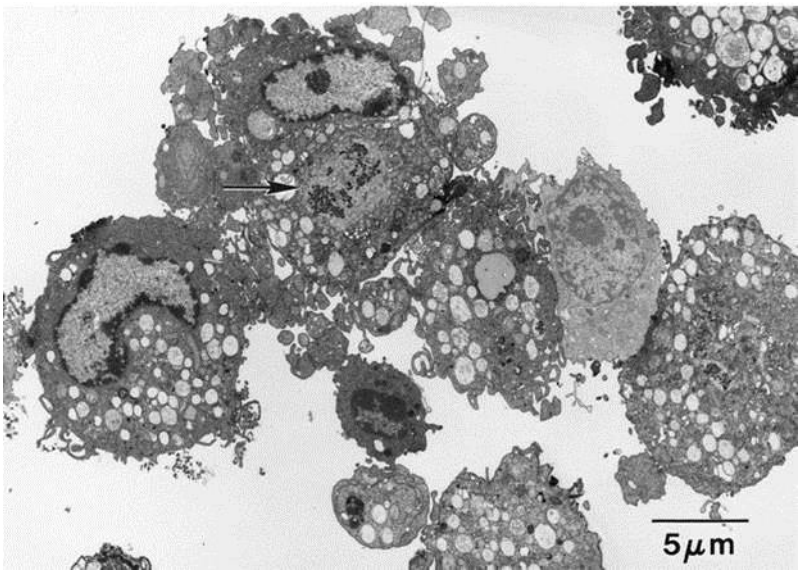

Figura 2: Macrófagos infectados por el vPPA aislado E70 a las 16 hpi. La flecha señala la localización de la factoría viral en el citoplasma. Fuente:(Zsak *et al.*, 2001)

Esta característica condiciona a su vez la forma de estudio del virus en el laboratorio, ya que hasta hace muy poco el aislamiento primario debía ser obligatoriamente realizado en monocitos o macrófagos. Esto suponía problemas de variaciones entre lotes, así como limitaciones debido a su falta de replicación en cultivo (de León *et al.*, 2013). Estos problemas se vieron parcialmente solventados con la adaptación de ciertos aislados vPPA al crecimiento en determinadas líneas celulares (VERO, MS, y CV) (Enjuanes *et al.*, 1976).

Sin embargo, el cambio más importante se produjo con la demostración de la utilidad de las células COS-1 para el aislado y titulación de aislados vPPA de campo (Hurtado *et al.*, 2010). Otras líneas celulares han sido posteriormente investigadas, observándose que tanto las células WSL como Sw Macde porcino, son susceptibles a la infección de cualquier aislado de vPPA, mientras que las IPAM son sensibles solamente a ciertos aislados del vPPA. El descubrimiento y uso de estas líneas celulares supone un avance importante en el estudio y diagnóstico del vPPA, ya que permiten amplificar la cantidad de virus presente en las muestras, preparar el antígeno necesario para los kits diagnósticos y optimizar los ensayos de placas para la titulación del vPPA de una forma más sencilla, barata y reproducible (de León *et al.*, 2013).

Debido a la ausencia de anticuerpos completamente neutralizantes, la clasificación de los aislados del vPPA, se realiza en base al genoma de los mismos. Para ello se analiza la región del extremo C-terminal del gen que codifica para la proteína vp 72, la proteína de mayor tamaño del

virus (Bastos *et al.*, 2003). En base a este análisis, a día de hoy se conocen 22 genotipos diferentes (Boshoff *et al.*, 2007). Todos ellos están presentes en el continente africano, siendo el genotipo I el que se encuentra más extendido en el continente. Mientras que algunos genotipos del vPPA permanecen restringidos a un solo país, otros están presentes en un gran número de países distintos. Es el caso del genotipo I, siendo el único genotipo presente en todos los países afectados del Oeste de África (Lubisi *et al.*, 2005). Fuera de África, el genotipo I fue el único implicado en todos los brotes ocurridos tanto en Europa como en América. Sin embargo, esto cambió en 2007, cuando se produjo la nueva entrada del vPPA en el Este de Europa, esta vez del genotipo II (Rowlands *et al.*, 2008).

Con el objetivo de conseguir una discriminación mayor, se han estandarizado protocolos de genotipado, incluyendo otras regiones de genoma, que permiten discriminar con mayor precisión grupos dentro del mismo genotipo. El estudio de la secuencia del gen de la proteína p54 permite diferenciar en subfamilias, y dentro de estas, el estudio de las repeticiones de la región central variable permite diferenciar grupos de aislados (Gallardo *et al.*, 2009a).

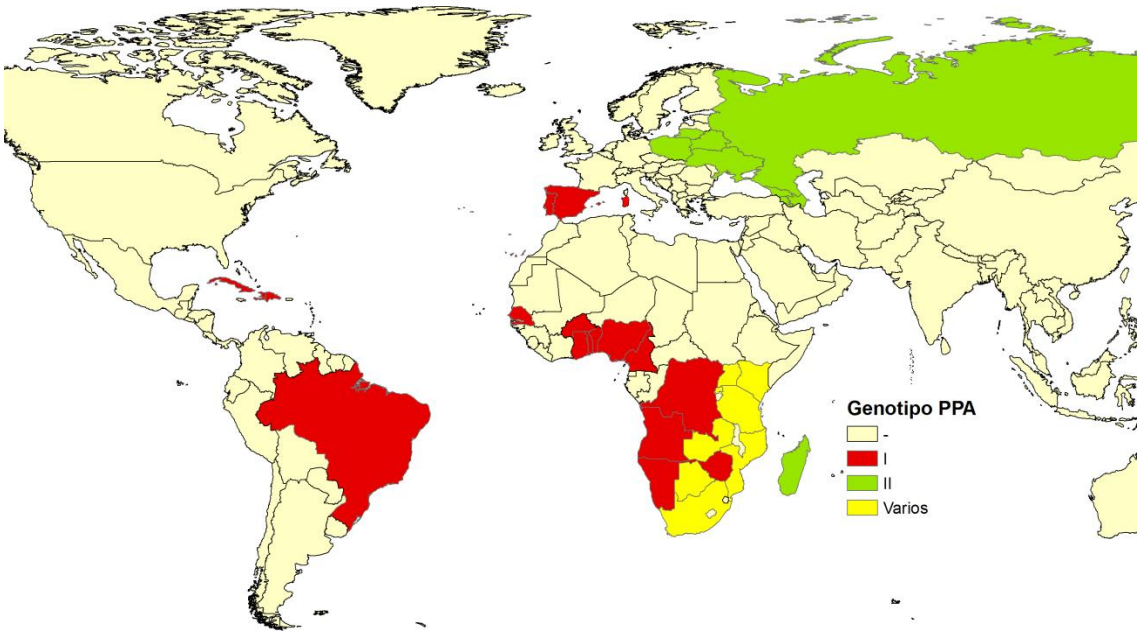

Figura 3: Representación esquemática de la distribución de los genotipos I y II de PPA en el mundo a lo largo de la historia. Fuentes: (Lubisi *et al.*, 2005, Rowlands *et al.*, 2008); elaboración propia).

No obstante, los métodos existentes en este momento presentan algunos inconvenientes ya que los genotipos establecidos no permiten predecir la protección cruzada entre aislados, ni proporcionan gran detalle sobre la filogenia (Chapman *et al.*, 2011). Del mismo modo, estudios realizados en Cerdeña, donde el virus lleva presente más de 30 años (Giammarioli *et al.*, 2011) y Rusia, tras 6 años de presencia (Malogolovkin *et al.*, 2012) no detectaron cambios en el genoma. Esto podría significar o bien una elevada estabilidad del virus, o la necesidad de buscar marcadores moleculares adicionales que permitan diferenciar más en detalle entre estos aislados. Por ello, se continúa investigando en esta línea, con el objetivo de encontrar otras regiones o métodos que permitan una discriminación más detallada de los aislados virales.

3.1.2. Hospedadores

Los únicos hospedadores del vPPA son los suinos. No es una enfermedad zoonótica, y su papel en la salud pública se limita a las consecuencias negativas ocasionadas en países en desarrollo por la pérdida de recursos, y en ocasiones, de la principal fuente de proteína animal (el cerdo).

Todos los suinos (familia Suidae) tanto domésticos como silvestres de todas las razas y edades son susceptibles a la infección del vPPA. Sin embargo, no todos sufrirán los síntomas y consecuencias negativas de la enfermedad. Mientras que en los cerdos domésticos y el jabalí europeo (*Sus scrofa*) la enfermedad cursa de forma sintomática, en los cerdos silvestres africanos la infección pasa por lo general desapercibida, sin mayores complicaciones para el animal infectado (Jori y Bastos, 2009). No obstante, existen diferencias entre las distintas especies y subespecies de suidos africanos respecto a papel en la epidemiología del vPPA, su localización geográfica y potencial contacto con el cerdo doméstico (Jori y Bastos, 2009).

Figura 4: Lechones de cerdo blanco (izquierda) y cerdos adultos de raza ibérica (derecha), ambos susceptibles al vPPA. Fuente: imágenes propias.

El facóquero común o cerdo verrugoso *Phacochoerus africanus*, ha sido considerado el hospedador vertebrado original del vPPA, que junto con *Ornithodoros porcinus* estableció el ciclo selvático ancestral (Penrith *et al.*, 2004b). Estos animales no presentan sintomatología alguna, y la replicación del vPPA sólo se da en animales jóvenes con inmunidad limitada. En muchos casos no se alcanzan niveles de viremia suficientes para la transmisión directa ya que replicación en sangre y tejidos de estos animales es considerablemente menor que en el cerdo doméstico (al menos dos logaritmos menos) (Thomson *et al.*, 1980, Oura *et al.*, 1998a). Por lo tanto, la transmisión del vPPA depende siempre de la presencia de garrapatas del género *Ornithodoros*, capaces de elevar exponencialmente la dosis viral y perpetuar el ciclo selvático del virus, presente en el Sur y Este de África.

El potamóquero (*Potamocherus larvatus y P. porcus*) ha sido siempre considerado de menor importancia en el ciclo de la PPA, ya que existe en menor número, y además la prevalencia de PPA en ellos es menor (Thomson, 1985). Los títulos de vPPA observados en potamóqueros infectados fueron suficientes para transmitir la infección a poblaciones susceptibles de cerdo doméstico. Sin embargo, no se transmitió la enfermedad a otros potamóqueros, lo que sugiere la posibilidad de que estas especies requieran mayores dosis de virus para infectarse en comparación con el cerdo doméstico (Anderson *et al.*, 1998). Su contacto con las garrapatas sería mucho más limitado debido a sus hábitos, ya que por lo general permanece alejado de las madrigueras donde estas se encuentran (Roger *et al.*, 2001).

Figura 5: Cerdos silvestres africanos hospedadores del vPPA. 1) Facóqueros (izquierda), potamóqueros (centro), e hialóquero (derecha). Fuentes: 1) cortesía de Carmina Gallardo), 2) The Telegraph, 3) Brighter future challenge.

Por último los hilóqueros (*Hylochoerus meinertzhangeni*) se localizan a grandes alturas en la región central de África (Huart y Yohannes, 1995), en zonas de bosque denso donde la presencia de cerdo doméstico no es habitual. Puede que esta sea una de las causas de su baja importancia en la epidemiología de la PPA (Jori y Bastos, 2009).

En el resto de continentes existen dos tipos de suidos silvestres con importancia en la epidemiología de la PPA: el jabalí Eurasiático, y el cerdo silvestre. La susceptibilidad de los jabalíes al vPPA ha sido ampliamente demostrada en infecciones naturales y experimentales, tanto con los aislados de la Península Ibérica (McVicar *et al.*, 1981, Perez *et al.*, 1998), como del Este de Europa (Blome *et al.*, 2013). Los jabalíes europeos presentan sintomatología de PPA y excretan el vPPA de forma similar al cerdo doméstico. Según la virulencia del aislado, se observará un porcentaje mayor o menor de supervivientes. En España, estudios serológicos confirmaron la presencia de anticuerpos en jabalíes, variando desde el 0,5% (Bech-Nielsen *et al.*, 1995) hasta el 10% en función del momento del muestreo (Perez *et al.*, 1998). En el Este de Europa, estudios preliminares sugieren la presencia de un bajo porcentaje de jabalíes seropositivos en zonas afectadas del Este de Europa (comunicación personal).

Varios estudios han evaluado el papel del jabalí europeo en el ciclo de la PPA, especialmente su potencial actuación como reservorio de la enfermedad. La hipótesis más aceptada a día de hoy, en base a los estudios realizados en Cerdeña (Laddomada *et al.*, 1994, Mannelli *et al.*, 1998) y España (Perez *et al.*, 1998), sugiere que en ausencia de reinfecciones por parte de cerdos domésticos o productos infectados, la enfermedad tiende a desparecer con el tiempo en la población de jabalíes. Sin embargo, esta hipótesis debería ser contrastada con

estudios que incluyesen otros factores relacionados con las características de las poblaciones de jabalíes, como la densidad y sobreabundancia de animales (Gortázar *et al.*, 2006), prácticas de manejo (comederos y bebederos), presencia de barreras naturales/artificiales, hábitat y disponibilidad de alimento, entre otros (Vicente *et al.*, 2007). Siendo todos ellos factores relacionados con la presencia y distribución de otras enfermedades animales en poblaciones silvestres.

Los cerdos domésticos son susceptibles a la infección por el vPPA y en general presentan sintomatología asociada a la infección. Sin embargo, en algunas zonas de África se ha detectado la presencia de PPA de forma endémica en poblaciones de cerdos domésticos aparentemente sanos con alta proporción de anticuerpos frente al vPPA, por lo que han sido considerados como potencialmente resistentes. Esto fue observado en Malawi (Haresnape *et al.*, 1985, 1987), Angola (Mendes, 1994), Zambia (Wilkinson *et al.*, 1988) y Mozambique (Penrith *et al.*, 2004a). Sin embargo, infecciones experimentales desarrolladas en estos animales demostraron una mortalidad cercana al 100%, incluso en animales con presencia de anticuerpos y vPPA en el momento del desafío. Estos resultados sugieren que la posible resistencia al vPPA en estos animales no es un factor heredable, sino más bien relacionado con el tipo/ruta/dosis de infección de los animales en su ambiente natural (Penrith *et al.*, 2004a).

Figura 6: Cerdos de razas locales africanas en sistema de traspatio. Fuente: imágenes propias.

El vPPA también es capaz de replicarse en garrapatas blandas, Argásidos, del género *Ornithodoros,* lo que las convierte en un componente importante del ciclo epidemiológico.

Las garrapatas del género *Ornithodoros* actúan como reservorios de la enfermedad, donde el vPPA persiste por largos periodos de tiempo (Sanchez-Bojita, 1963). En ellos, el vPPA puede permanecer infeccioso hasta cinco años (Boinas *et al.*, 2011). En el continente africano se conoce el papel importante de *O. moubata* junto con los facóqueros en el ciclo selvático de PPA en el Sur y Este de África (Plowright *et al.*, 1969, Haresnape y Mamu, 1986). Esta especie es capaz de infectar a sus congéneres tanto de forma tras-ovárica, trans-estádica, como sexual de machos a hembras (pero no viceversa), facilitando el mantenimiento y difusión de la infección (Plowright *et al.*, 1970, Rennie *et al.*, 2001, Thomson, 1985).

En la Península Ibérica, la presencia de *O.erraticus* ha sido ampliamente demostrada, así como su importante papel en la persistencia de la enfermedad (Sanchez-Bojita, 1963, Oleaga-Perez *et al.*, 1990, Perez-Sanchez *et al.*, 1994). Sin embargo, a diferencia de la especie africana, *O.erraticus* sólo es capaz de transmitir la infección de forma trans-estádica (Caiado *et al.*, 1988).

Figura 7: Fotografía de un ejemplar de *Ornithodoros moubata* (izquierda) y dos ejemplares de *O.erraticus* (derecha). Fuentes: izq: Natural History Museum, London/Science Photo Library; dcha: Ricardo Pérez, IRNASA (via dicyt.com).

En Europa, tanto en la cuenca del Mediterráneo como en los países del Cáucaso y la Federación Rusa, las únicas especies de *Ornithodoros* detectadas hasta la fecha pertenecen al complejo *O. erraticus* (*O. alactagalis, O. asperus, O. normandi, O. pavlovskyi, O. tartakovskyi,O. tholozani, O.erraticus, O.lahorensis y O.sonrai)*. El conocimiento sobre la distribución y preferencias

de hábitat y hospedador de estas especies es limitada. Estudios de campo realizados en Cerdeña en los años 1980 sugieren la ausencia de *O.erraticus* en la isla (Ruiu A, 1989). No obstante, considerando que todas las especies de *Ornithodoros* analizadas hasta la fecha resultaron susceptibles a la infección del vPPA, es de vital importancia conocer la distribución y competencia de estas especies para la transmisión de PPA (EFSA, 2010b).

La presencia/ausencia de los distintos hospedadores y garrapatas, así como su participación en la transmisión y difusión del vPPA en los distintos territorios afectados, da lugar a la existencia de distintos ciclos epidemiológicos. Los detalles y características epidemiológicas respecto a los distintos ciclos epidemiológicos se encuentra recogidos en la primera publicación de la tesis (*ASF epidemiological update*- Objetivo 1).

3.1.3. Transmisión

El vPPA se transmite tanto de forma directa por medio de las secreciones contaminadas de los animales infectados a los susceptibles, como indirecta a partir de materiales/productos contaminados, así como la infección por picadura de garrapata, anteriormente comentada.

Una vez producida la entrada del vPPA en el organismo, el primer punto de replicación serán los macrófagos y monocitos de los ganglios linfáticos más cercanos al punto de entrada. Frecuentemente, la infección se producirá por vía oro-nasal, siendo los ganglios sub-mandibulares y tonsilas el primer punto de replicación del virus. Tras esta fase inicial, el vPPA se distribuye a través de la sangre y/o sistema linfático, por lo que a los días 4-8 post-infección (pi) es frecuente encontrar virus en sangre, detectable durante semanas o meses. A continuación, el vPPA viajará a través del torrente sanguíneo y linfático a los sitios secundarios de replicación, localizados principalmente en nódulos linfáticos, bazo, pulmón, hígado, médula ósea y riñón (Sánchez-Vizcaíno y Arias, 2012).

En estos órganos y tejidos, el vPPA puede permanecer presente durante largos periodos de tiempo, convirtiéndose en fuente de contaminación de otros animales (Wilkinson, 1984).

El vPPA se transmite a partir de todas las secreciones de los animales infectados, desde los días 1 a 7 pi. Esto variará en función del aislado viral, la ruta de infección, edad y estado sanitario e

inmunitario del animal infectado. Los fluidos oro-nasales suelen presentar títulos más altos de vPPA que otras excreciones, especialmente durante la fase clínica de la enfermedad. En otras excreciones como la genital, conjuntiva o las heces, la cantidad de vPPA es menor y la excreción viral es a menudo intermitente (de Carvalho Ferreira *et al.*, 2012, Greig y Plowright, 1970, Ekue *et al.*, 1989).

El fluido más importante para la difusión del vPPA es, sin lugar a dudas, la sangre de los animales infectados, en la que el vPPA se asocia a los eritrocitos (Figura 8) (Quintero *et al.*, 1986).

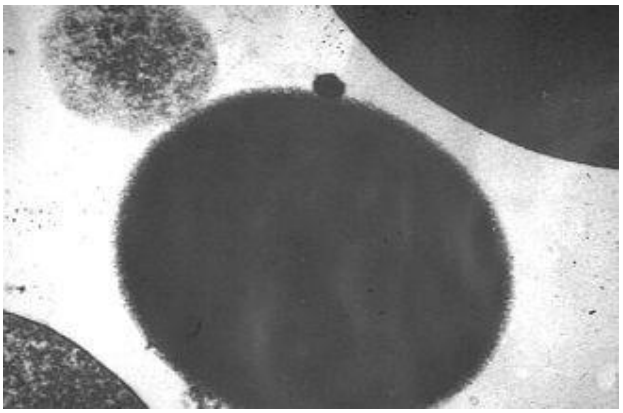

Figura 8: Imagen de microscopia electrónica de un vPPA asociada a la membrana de un eritrocito. Fuente: J. Quintero.

Las dosis de virus en sangre son considerablemente superiores al del resto de secreciones, por lo que una pequeña cantidad de sangre sería capaz de infectar a un número considerable de animales. En 200 microlitros de sangre tras infección con vPPA del Cáucaso, se detectaron $3*10^6$ copias de DNA (Gabriel *et al.*, 2011, Blome *et al.*, 2013). Además, el periodo de viremia en PPA es muy prolongado, posiblemente debido a la falta de neutralización del virus mediante anticuerpos neutralizantes, lo que permite la presencia prolongada del vPPA en el torrente sanguíneo de los animales infectados. Experimentos recientes realizados con aislados moderadamente virulentos del genotipo I confirmaron estos hechos, así como la existencia de animales persistentemente infectados durante al menos 70 días, con potencial de excreción viral (de Carvalho Ferreira *et al.*, 2012). Por ello cualquier manifestación clínica que suponga presencia de sangre (epistaxis, melena, sangrados inespecíficos…) supone un alto riesgo para la difusión del vPPA.

Respecto a la transmisión indirecta, cabe destacar que el vPPA es un virus muy estable, siempre y cuando el pH del medio se encuentre entre 4 y 10. Por encima o por debajo de ese pH se

inactiva en escasos minutos (EFSA, 2010a). Por medio de temperatura es posible lograr su inactivación cuando se alcanzan los 60ºC durante al 20 minutos o los 56ºC durante 70 minutos (OIE, 2014a). Por ello, si no se realizan las tareas de limpieza y desinfección adecuadas, el vPPA es capaz de permanecer infeccioso en granjas contaminadas más de tres días y varias semanas en heces de cerdo (EFSA, 2014).

A su vez, el vPPA permanece presente y activo en un gran número de productos cárnicos producidos a partir de cerdos infectados durante largos periodos de tiempo. Por ejemplo, en carne contaminada conservada en refrigeración, el vPPA permanece infeccioso hasta 300 días, mientras que si permanece congelada, resiste hasta 1000 días (Adkin *et al.*, 2004). En otro tipo de productos cárnicos, elaborados mediante distintos procesos de secado y salazón como el jamón y lomo serrano, estos periodos de supervivencia del vPPA fueron menores al tiempo requerido para el procesado (Mebus *et al.*, 1993, Mebus *et al.*, 1997). Sin embargo, otras variedades de embutidos, salchichas y productos que han sufrido un procesados y tratamientos distintos, será necesario su estudio de manera individual, ya que no es posible extrapolar condiciones tan distintas (EFSA, 2014).

Este hecho determina una de las características más significativas del vPPA en cuanto a su transmisión y difusión. La larga permanencia del vPPA en los tejidos contaminados, unido a su gran resistencia a las distintas condiciones de temperatura, son claves para que la principal vía de entrada de la enfermedad en países anteriormente libres fuese **la entrada por desperdicios de productos de cerdo contaminados, transportados en barcos o aviones internacionales** (Sánchez-Vizcaíno y Arias, 2012). Esta fue la vía de entrada en Portugal (1957 y 1960), Bélgica, Malta, Cerdeña, Brasil, República Dominicana y Sao Tomé y Príncipe (Sánchez-Botija, 1982).

Una vez la enfermedad se ha establecido en una zona, la transmisión por contacto directo entre animales infectados y sanos adquiere gran relevancia. Otras formas de transmisión frecuentes incluyen los productos elaborados con animales infectados anteriormente mencionados, los vehículos contaminados, el personal (veterinario y no veterinario) contaminado, la comida, material quirúrgico/sanitario, así como cualquier tipo de material que sirva para transportar el virus.

Además del papel de las garrapatas del género *Ornithodoros* que actúan como vectores de la enfermedad, se conoce la implicación que otros vectores que pueden transmitir el vPPA de

forma meramente mecánica. En el caso de las moscas *Stomoxys calcitrans* se demostró la transmisión de vPPA a animales susceptibles tras 24 horas, sin observarse disminución del título viral en 48 horas (Mellor *et al.*, 1987). Por lo que se asume que estas moscas serían capaces de transmitir el vPPA por transporte de material (sangre o fluidos contaminados) en sus patas durante dos días (Baldacchino *et al.*, 2013).

Cabe destacar que, si bien el vPPA no presenta unos mecanismos de transmisión tan eficaces como otros virus (véase Fiebre aftosa y los aerosoles), la experiencia histórica demuestra que estos son más que suficientes para asegurar su difusión, siempre y cuando las medidas de bioseguridad y control no sean las adecuadas.

3.1.4. Síntomas y lesiones

La infección del vPPA causa una serie de síntomas y lesiones, consideradas habitualmente de tipo congestivo-hemorrágico, si bien esto varía en gran medida en función de la especie afectada, el aislado viral implicado, la ruta y dosis de infección. El periodo de incubación de la PPA varía entre los 4 y 19 dpi (EFSA, 2010a), a partir de los cuales los animales infectados comenzarán a presentar los signos clínicos de la enfermedad. Normalmente, la morbilidad suele variar entre el 40 y 85%, mientras que las tasas de mortalidad varían ampliamente en función de la virulencia del aislado implicado, pudiendo alcanzar valores cercanos al 100% (EFSA, 2010a).

Se han descrito distintas formas clínicas de la PPA, desde la forma hiperaguda en la cual sólo observamos la muerte repentina de los animales; la forma aguda y subaguda e incluso una forma crónica observada en la Península Ibérica. Las formas más frecuentemente observadas a día de hoy en la naturaleza son las *formas aguda y subaguda* de la enfermedad. En ellas, el primero de los síntomas observados es la aparición de fiebre elevada, inapetencia, postración, acompañado de eritemas cianosis en piel, especialmente en orejas, abdomen y extremidades (Sánchez-Vizcaíno y Arias, 2012). En el curso hiperagudo es bastante probable que se produzca la muerte del animal sin ningún otro síntoma más que los anteriormente descritos. La tasa de mortalidad en los animales infectados es bastante elevada, muriendo la mayoría de animales afectados entre los días 5 y 12 pi (EFSA, 2010a).

En la *forma subaguda*, causada habitualmente por aislados atenuados o de menos virulencia, el curso de la enfermedad es más lento y permite la aparición de síntomas y lesiones más evidentes, incluyendo la aparición de diarrea con o sin hemorragia, descarga nasal, tos, disnea, epistaxis, abortos en cerdas preñadas, o incluso síntomas nerviosos como paresia de las articulaciones posteriores, ataxia, crisis convulsivas con contracciones tónicas y clónicas. Estos síntomas también se pueden observar en la forma aguda, aunque con menor frecuencia.

Figura 9: Fotos de cerdos afectados por PPA en los que observamos decaimiento y agrupación de los animales debido a las temperaturas elevadas (a) y ligero enrojecimiento del pabellón auricular (b). Fuente: imágenes propias.

La *forma crónica* no se presenta a día de hoy en la naturaleza, habiendo sido únicamente observada en España y Portugal durante los años de presencia de la enfermedad (1960-1995). En esta forma, caracterizada por la mayor supervivencia, los animales infectados presentan fiebre irregular y ondulante, inapetencia, neumonía acompañada de tos, retraso en el crecimiento y artritis. Pueden aparecer lesiones (nódulos, úlceras y necrosis) en las orejas, jeta, tronco y articulaciones. Se pensaba que esta forma era una evolución de la enfermedad causada por aislados de baja virulencia o la recuperación de los animales que sufrieron la forma aguda (Sánchez-Botija, 1982). Sin embargo, en otras zonas del planeta donde la PPA permanece presente de forma endémica desde hace más de 35 años (Cerdeña) y casi un siglo en África, no se han observado nunca unos síntomas similares. Estudios recientes han demostrada la similitud genética de los aislados virales de la época con la de la vacuna atenuada desarrollada y empleada en campo tanto en España y Portugal (Manso-Ribeiro *et al.*, 1963). Si bien, esto no ha podido ser del todo demostrado, se sospecha que las formas crónicas de la PPA pudieron ser causadas por unos

aislados vacunales más que por un virus de campo, lo que descartaría la forma crónica como un curso natural de la enfermedad.

Las lesiones más típicas observadas en los animales afectados por PPA son aquellas relacionadas con las hemorragias y destrucción del tejido linfoide (Ekue *et al.*, 1989), localizadas principalmente en nódulos linfáticos, bazo, riñón y corazón. Sin embargo, estas lesiones son muy escasas o ausentes en los animales afectados por la forma crónica de la enfermedad anteriormente comentada (Sánchez-Vizcaíno y Arias, 2012). La lesión más frecuentemente observada es la hiperplasia y hemorragia de los nódulos linfáticos, especialmente los gastro-hepáticos y renales (Blome *et al.*, 2013). La esplenomegalia es también muy común, presentando el bazo un aspecto oscuro y friable al tacto, con presencia de infartos y focos de necrosis, recibiendo el nombre de "esplenomegalia infartada".

En la cavidad torácica es frecuente observar hidrotórax, edema y lesiones hemorrágicas en pulmón (conviene diferenciar de lesiones de sacrificio), hidropericardio con abundante líquido sero-hemorrágico, petequias y equimosis en corazón. En cavidad abdominal los riñones presentan a menudo petequias en la cortical y pelvis renal, y el hígado y la vesícula biliar congestión y petequias. Es común la presencia de ascitis con presencia de exudado sero-hemorrágico. Otras lesiones se pueden observar en la vejiga de la orina, intestino (petequias), así como congestión en las meninges y encéfalo (Sánchez-Vizcaíno y Arias, 2012).

No obstante, a pesar de las lesiones hemorrágicas, los síntomas y lesiones de la enfermedad no se muestran siempre de una forma tan evidente y, en muchos casos no es tan sencillo diferenciarlo. Por ello, será necesario realizar siempre un **diagnóstico diferencial** especialmente con otras enfermedades hemorrágicas del cerdo como la peste porcina clásica, salmonelosis aguda, mal rojo (*Erysipela),* así como intoxicaciones alimentarias que pueden provocar cuadros hiper-agudos con alta mortalidad.

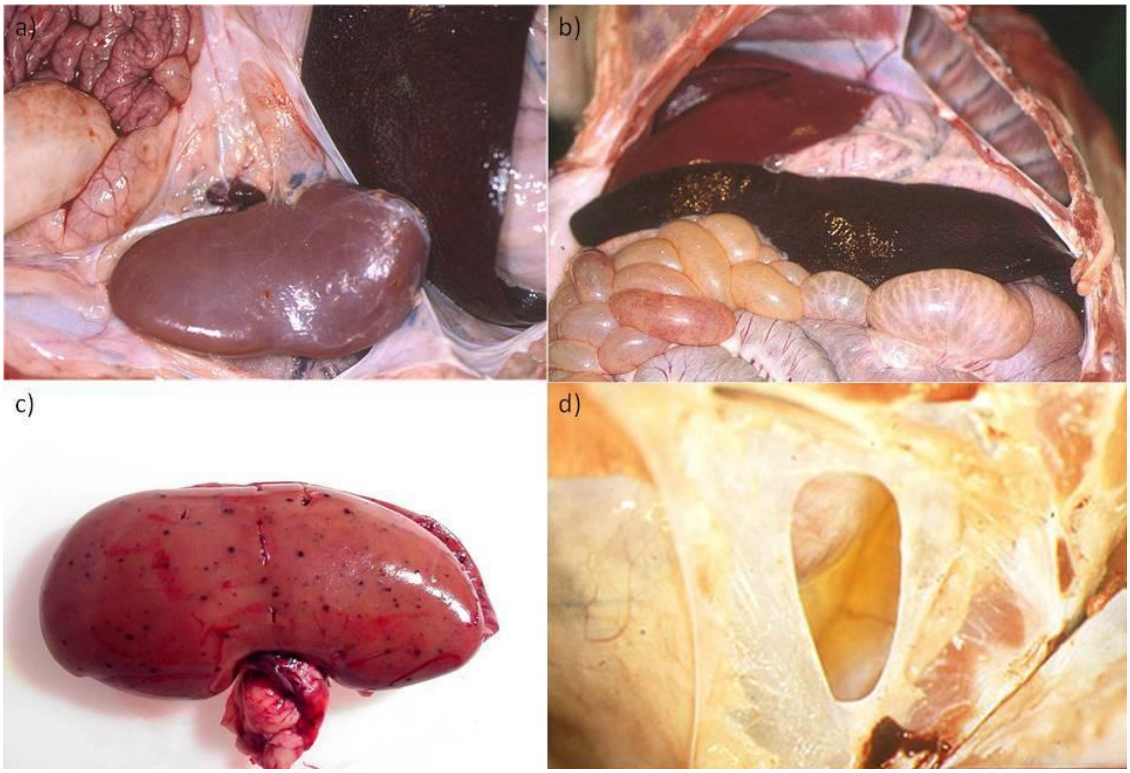

Figura 10: Imagen de algunas de las lesiones más frecuentemente observadas en PPA. a) nódulo linfático renal hemorrágico, b) bazo aumentado de tamaño y de un color oscuro, c) presencia extensiva de petequias en la superficie del bazo, d) hidrotórax. Fuente: SUAT, VISAVET.

3.1.5. Diagnóstico

Teniendo en cuenta la gran similitud que pueden presentar los síntomas clínicos de PPA con otras enfermedades porcinas como PPC, salmonelosis aguda o mal rojo, siempre es necesario un diagnóstico laboratorial. Además, cabe destacar que la presentación de la enfermedad en el campo no es siempre es exacta a lo descrito en los libros y tratados de enfermedades infecciosas, basados a menudo en infecciones experimentales. Es posible que en el campo, los signos puedan ser confundidos fácilmente con otras enfermedades. Por lo que, ante una sospecha de PPA, se deberán realizar las pruebas diagnósticas necesarias que permitan descartar o confirmar el foco.

Actualmente existe una gran variedad de técnicas diagnósticas correctamente desarrolladas y validadas para el diagnóstico laboratorial de la PPA, incluyendo todos los genotipos existentes. En el curso de PPA, las viremias son muy prolongadas, debido a la ausencia de anticuerpos neutralizantes, lo que facilita la detección del virus en animales infectados. Del mismo modo, los anticuerpos producidos por la infección del vPPA son signo inequívoco de infección previa y persisten durante largos periodos de tiempo en el animal, por lo que las pruebas serológicas

deberían realizarse siempre en paralelo a los métodos de detección de antígeno (Sánchez-Vizcaíno y Mur, 2013).

Para la detección del VPPA, la técnica más ampliamente empleada a día de hoy es la PCR (Reacción en Cadena de la Polimerasa), por su rapidez, alta sensibilidad y posibilidad de uso incluso en muestras mal conservadas. Hay dos tipos de PCR actualmente aprobados por la OIE para el diagnóstico de PPA; una PCR convencional y otra a tiempo real, ambos dirigidos a amplificar una región conservada del gen de la vp 72 (Agüero *et al.*, 2003, King *et al.*, 2003).

El aislamiento viral en macrófagos unido a la técnica de hemoasdorción (Malmquist y Hay, 1960) es la técnica de referencia para el aislamiento e identificación del vPPA , aunque su uso está restringido a laboratorios de referencia o ante el caso de un primer foco en un país libre. Otras técnicas directas como la inmunofluorescencia directa (Bool *et al.*, 1969) o el ELISA de captura de antígeno se usan actualmente con menor frecuencia, si bien continúan disponibles y en uso en ciertos países.

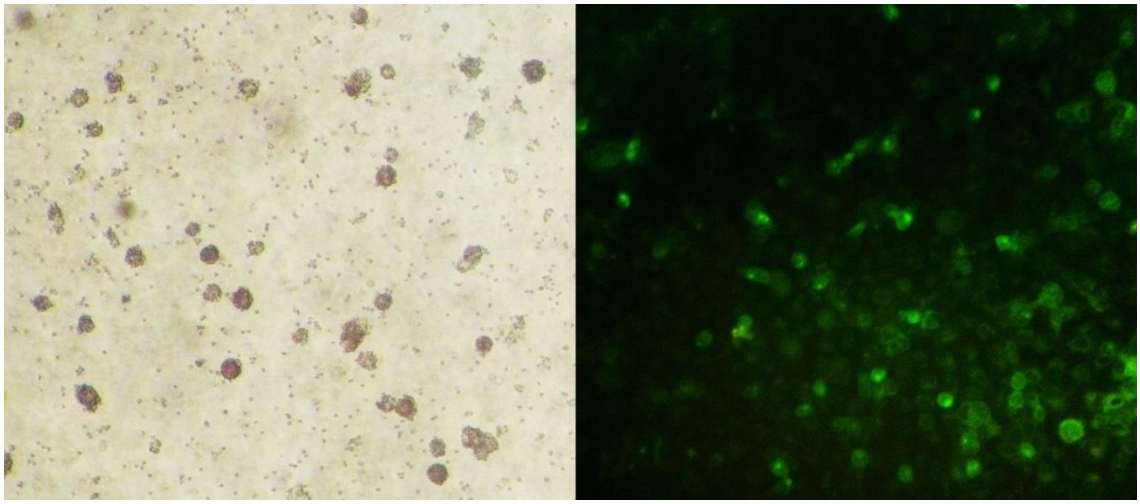

Figura 11: Imagen del fenómeno de hemoadsorción en macrófagos pulmonares de porcino infectados con el vPPA (izquierda) y de una impronta de tejido positivo a la técnica de inmuno-fluorescencia directa empleando un anticuerpo monoclonal frente a la proteína vp 72 del vPPA (derecha). Fuente: imágenes propias.

En el caso de la detección de anticuerpos, la técnica más empleada es el ELISA (Enzyme-linked-immunosorbent assay) (Sanchez-Vizcaino *et al.*, 1979, Sanchez-Vizcaino *et al.*, 1982), ya que permite analizar gran cantidad de muestras en un breve periodo de tiempo, a un coste asequible. Esta es la técnica prescrita por la OIE para regular el comercio internacional (OIE, 2012a). Además

del ELISA conocido como de la OIE, que emplea una antígeno soluble del virus (Sanchez-Vizcaino *et al.*, 1982), existen ELISAs comerciales disponibles. Los resultados del ELISA pueden ser confirmados por otro tipo de técnicas, entre las que se incluyen la inmunofluorescencia indirecta (Bool *et al.*, 1969), el inmunoblotting (Pastor *et al.*, 1989) o la técnica de inmunoperoxidasa (Pan *et al.*, 1978). A día de hoy, estas técnicas desarrolladas hace ya algunos años continúan funcionando adecuadamente con todos los genotipos del vPPA circulantes, tal y como se demostró con una comparación intensa empleando técnicas basadas en el uso de antígenos específicos (Gallardo *et al.*, 2013).

Las muestras empleadas habitualmente para el diagnóstico de PPA son sangre y suero de los animales sospechosos y los órganos diana obtenidos durante la necropsia, entre los que destacan el bazo, ganglios linfáticos, pulmón, hígado, riñón, médula ósea y corazón. Recientemente, y como objetivo de esta Tesis, se han puesto a punto técnicas serológicas para el diagnóstico serológico de PPA en muestras de fluido oral (Mur *et al.*, 2013), demostrando la utilidad de éste como muestra útil para el diagnóstico de esta enfermedad.

3.1.6. En búsqueda de una vacuna

A pesar de los numerosos esfuerzos realizados en esta área, en la actualidad no contamos con una vacuna eficaz para la prevención de la PPA.

Los primeros intentos se realizaron con vacunas atenuadas empleadas en campo en la Península Ibérica en campo durante los años 60, provocando aparentemente la aparición de una forma crónica de la enfermedad con graves lesiones en piel y articulaciones, así como alteraciones respiratorias (Manso-Ribeiro *et al.*, 1963). Este tipo de protección cruzada se observó también con aislados del vPPA de campo de baja virulencia, como OURT o NHV (Leitao *et al.*, 2001, Boinas *et al.*, 2004, King *et al.*, 2011). Sin embargo, al igual que los intentos anteriores, presentaron ciertos problemas de seguridad y ausencia de protección total frente a vPPA heterólogos (Ruiz-Gonzalvo *et al.*, 1981).

Por ello, se continúan investigando otras líneas de acción con el intento de reducir la virulencia del VPPA, evitando así las reacciones adversas de la vacunación. Entre ellas se incluyen las vacunas basadas en vPPA mutantes deleccionados no infecciosos (Lewis *et al.*, 2000, Salguero *et al.*, 2008), las vacunas de subunidades y de DNA. No obstante, a día de hoy, los virus atenuados siguen siendo la alternativa que ha proporcionado datos más interesantes, demostrando que tanto

los anticuerpos, como la respuesta celular mediada por linfocitos citotóxicos T-CD8 tienen un papel muy importante en la protección frente al vPPA.

A pesar de estos avances realizados, es necesario continuar investigando todas las opciones con el objetivo de obtener una vacuna adecuada para la prevención y control de la enfermedad. Sin embargo, la experiencia de España, Portugal y otro países como Brasil, República Dominicana y Haití, demuestran cómo tras más de 30 años con PPA, es posible lograr la erradicación sin una vacuna disponible.

3.2.EVOLUCIÓN EPIDEMIOLÓGICA DE LA PPA

3.2.1. África

La PPA fue descubierta en 1921 por Montgomery en Kenia, cuando al introducir cerdos domésticos europeos en el continente africano se empezaron a observar altas tasas de mortalidad (Montgomery, 1921). Al inicio se pensó que podría tratarse de PPC, pero pronto se vio que el agente causal era diferente. La introducción de las razas europeas en África durante el siglo XX, con el objetivo de facilitar la producción de proteínas, supuso un incremento exponencial del número de cerdos en el continente. En menos de 60 años el número de cerdos en África se multiplicó por cinco (FAO, 2014).

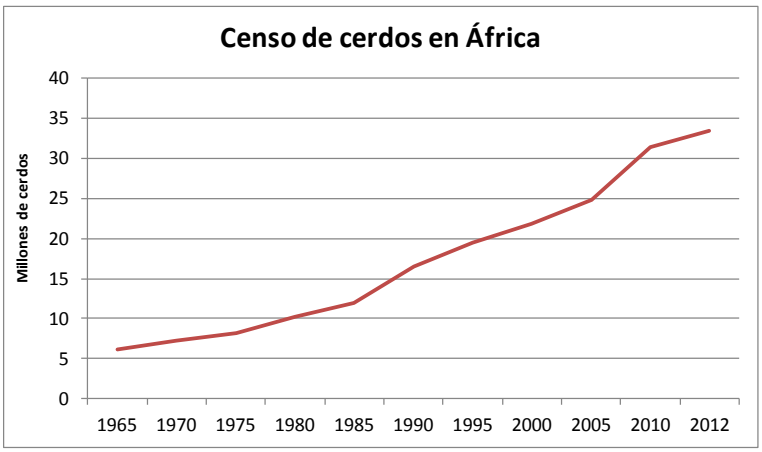

Figura 12: Gráfico que representa el importante crecimiento de la producción de cerdo en el continente africano. En el eje de las X los años de estudio (1965-2012), en el eje de las Y los millones de cerdos presentes en África. Fuente datos: (FAO, 2014), elaboración propia.

Este importante crecimiento de la producción porcina, sumado a la intensificación del transporte y comunicaciones, facilitaron que la PPA se extendiese por todo el continente, alcanzado primero el Sur (Sudáfrica en 1928) y Este de África (Angola, 1932), afectando a continuación a los países centroafricanos (Penrith y Vosloo, 2009). La primera noticia de la presencia de la PPA en el Oeste de África data de 1959, cuando el genotipo I de vPPA se detectó en cerdos domésticos de Senegal (Etter *et al.*, 2011). Pasaron once años hasta que un nuevo brote ocurrió en Camerún en 1982, seguido de un segundo en 1985, lo que destrozó casi por completo la industria porcina del país, que se encontraba en fase de crecimiento (Nana-Nukechap y Gibbs, 1985).

Sin embargo, fue a partir de 1994 cuando la PPA inició su época de máxima expansión en el continente africano (Penrith *et al.*, 2004b). En ese año la PPA alcanzó las capitales de dos países del Este de África, Mozambique y Kenia, causando importantes daños a la industria porcina cercana a Maputo y Nairobi (Penrith y Vosloo, 2009). Dos años más tarde, en 1996, la PPA llegó al oeste africano, primero a Costa de Marfil, desde donde alcanzó Gambia, Senegal, Benín, Togo, y Nigeria en los años sucesivos (Owolodun *et al.*, 2010). Otros países, más recientemente afectados son Burkina Faso y Chad y las islas del continente, como Madagascar, Mauricio y Cabo Verde. En la mayoría de estas zonas la PPA continúa siendo endémica.

A día de hoy, la PPA se encuentra presente de manera oficial en 21 países del continente africano, incluyendo tanto las formas clínicas de enfermedad, como la presencia de infección sin clínica o los países limitados a ciertas zonas. Esto supone un 45% de los países de África subsahariana, abarcando una gran extensión de territorio en el continente (Figura 13). Otros seis países registraron brotes en el pasado y hay 10 países de los cuales no se tiene información respecto a la situación de la PPA (OIE, 2014b). Durante el año 2012, los países africanos que sufrieron más las consecuencias de la enfermedad fueron la República Democrática del Congo (80 brotes) y Camerún (66 brotes) (AU-IBAR, 2012). Si bien, existen diferencias entre ellos, ya que como se explica en el artículo "*ASF epidemiological update*", existen diferentes ciclos epidemiológicos de PPA, no todos ellos presentes en todos los lugares donde la enfermedad está presente.

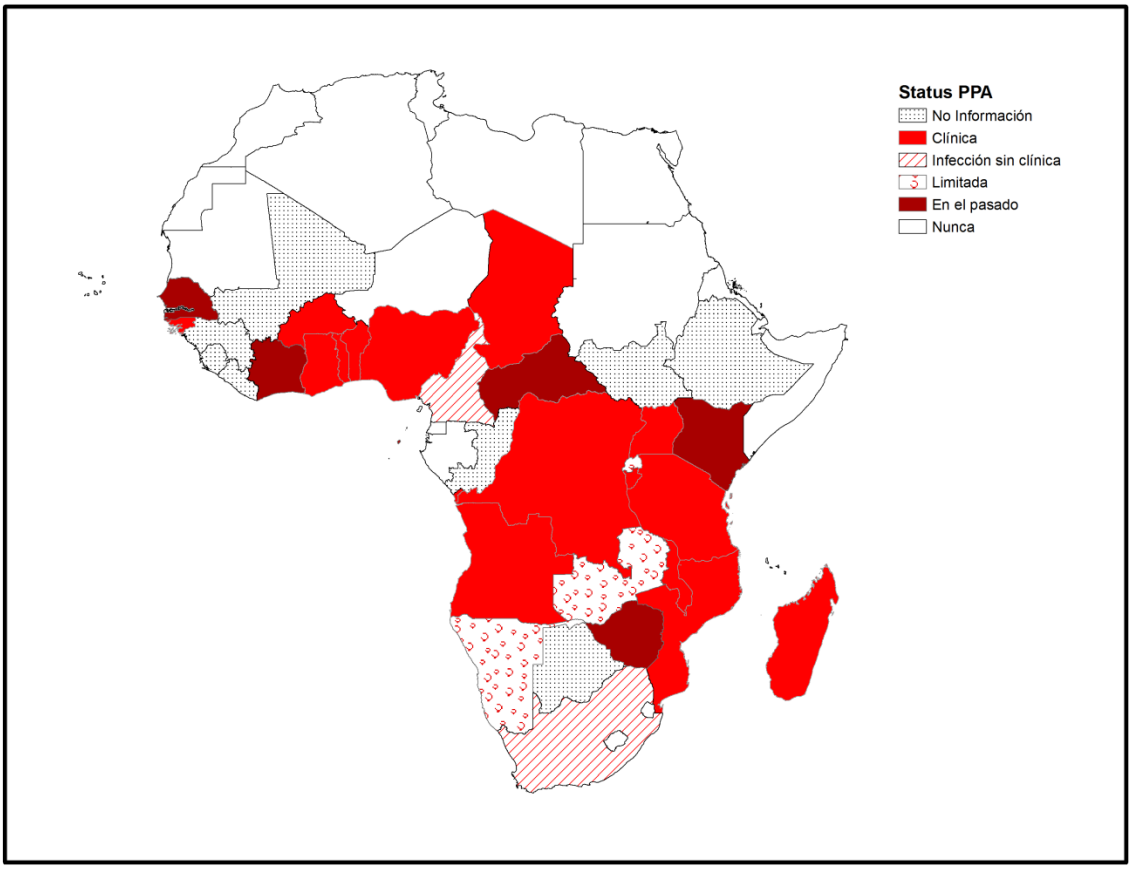

Figura 13: Mapa de África representando el status oficial de PPA según datos de la OIE 2014 (Fuente: OIE-Wahid, elaboración propia).

No obstante, a pesar de que la PPA es endémica en un gran número de países de África subsahariana, ha adquirido la relevancia que presenta actualmente debido a las **tres incursiones realizadas fuera de África**. Dos de las cuales, no han logrado ser todavía controladas y con importantes consecuencias negativas.

3.2.2. Península Ibérica y brotes relacionados

La primera salida del vPPA de la que se tiene constancia ocurrió en 1957 cuando unos casos de PPA se notificaron en Portugal, en una granja muy cerca de Lisboa, probablemente por la llegada de comida contaminada del continente africano. Tras el sacrificio de más de 10.000 cerdos, este foco fue rápidamente y eficazmente controlado (Manso-Ribeiro *et al.*, 1958).

Sin embargo, tres años más tarde, en 1960 el vPPA volvió a entrar en Portugal, difundiéndose esta vez por todo el país y alcanzando rápidamente España. Justo en aquellos años la industria porcina empezaba a despuntar, y la producción de tipo familiar fue dando paso progresivamente a sistemas intensivos, con la incorporación de razas europeas y nuevos métodos de manejo. Del mismo modo, la localización de estas granjas varió en el país, apareciendo nuevas granjas industriales en regiones alejadas de la zona suroccidental tradicional (Anónimo, 1987). En este contexto de cambio y desarrollo, llegó la PPA a España, afectando tanto al cerdo doméstico como a los jabalíes. La PPA ocasionó importantes costes en España siendo un importante problema para la industria ya que no se podían exportar animales ni productos. Pero a pesar de ello, la industria porcina continuó su proceso de desarrollo, incrementando notablemente los censos. Se pasó de 6 millones de cerdos en 1960 a 16,7 millones en 1989 (Arias y Sánchez-Vizcaíno, 2002).

Tras más de 15 años de presencia relativamente estable del vPPA en la Península Ibérica, en 1977 la enfermedad sufrió una recrudescencia importante tanto en España (1780 explotaciones afectadas en 1978) como en Portugal (5017 explotaciones afectadas en 1978). Ambos países sufrieron en ese año las mayores incidencias de PPA registradas hasta el momento. Este aumento de la enfermedad coincidió, pudiendo ser una de las causas de ello, con la aparición de brotes de PPA en 1978 en Malta, Cerdeña, Brasil, República Dominicana, Haití, y Cuba donde hubo un segundo brote en 1980 (Sánchez-Botija, 1982). En muchas de estos países afectados por primera vez por PPA, se implementaron medidas drásticas de control que incluyeron la despoblación total de cerdos en el territorio (Malta, República Dominicana, una provincia de Cuba (Guantánamo)). De esta forma se logró erradicar la enfermedad en breves periodos de tiempo.

Además de las salidas del año 1978, el vPPA no se limitó a afectar a España y Portugal en Europa, sino que se produjeron numerosos focos en países libres, la mayoría de ellos infectados por desechos de carne contaminada empleados en la alimentación de los cerdos. Algunos de los países europeos afectados por la PPA fueron Bélgica (1985), Holanda (1986), Francia (1964), Italia (1967, 1969 y 1993) y Malta (1978). De todas estas zonas afectadas por el genotipo I del vPPA, la enfermedad fue eficazmente controlada y erradicada, siendo a día de hoy, Cerdeña, el único territorio donde permanece endémica desde su entrada en 1978.

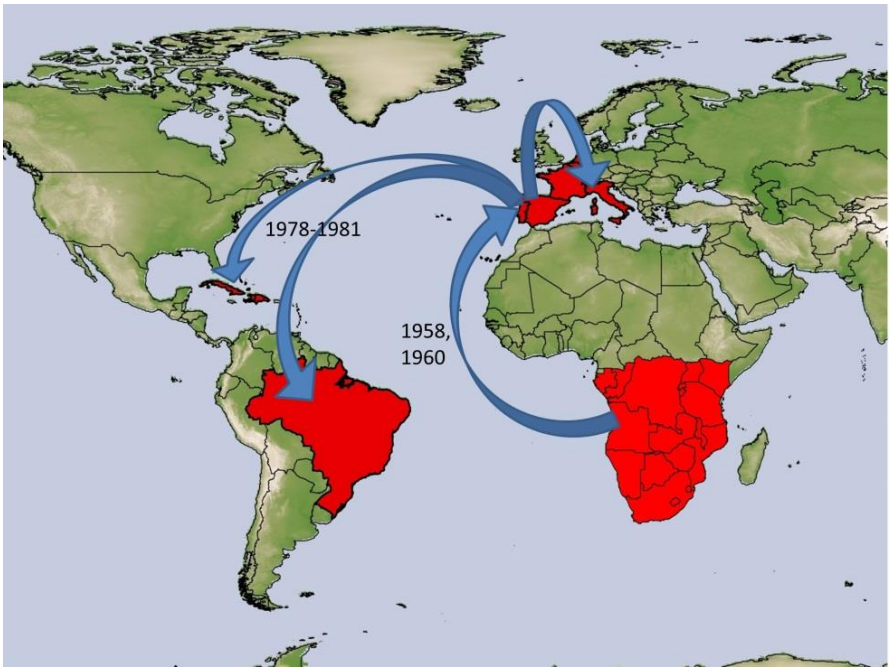

Figura 14: Representación de la evolución de la PPA en el mundo desde 1958 a 1990 (Fuente: OIE-Wahid, elaboración propia)

Tras la recrudescencia del año 1977 en la península Ibérica, los focos de PPA iniciaron a controlarse gracias a las políticas de control implementadas. El número de brotes de PPA continuó descendiendo en España desde 1977 hasta 1981, año en que comenzó una nueva ola epidémica. Esa nueva ola duraría hasta 1984, alcanzando su punto máximo con los 1031 focos detectados en 1984 (Sanchez-Vizcaino, 1988).

A partir de 1985 se intensificaron las actividades del nuevo programa de erradicación en España cofinanciado por la Unión Europea, que comprendía en un principio hasta 1990, pero fue prorrogado hasta 1994. Gracias a los esfuerzos realizados, en 1989 se logró la regionalización de España, diferenciando entre zona afectada (comprendía el suroeste del país) y zona indemne para el resto de España. A partir de ese momento, desde la zona indemne se producían exportaciones tanto de animales vivos como de sus productos con total normalidad. Más adelante, se diferenció una tercera zona de vigilancia, hasta que finalmente en el año 1995 se declaró oficialmente la PPA erradicada de España, siguiendo el paso de Portugal que logró erradicarla un año antes (1994) (Gomez-Tejedor, 1993).

Durante los años de presencia del VPPA en la Península Ibérica, se realizaron numerosos avances en el diagnóstico de PPA, entre los que destaca la técnica de ELISA para la detección de

anticuerpos (Sanchez-Vizcaino *et al.*, 1979, Sanchez-Vizcaino *et al.*, 1982). También fue de vital importancia el conocimiento adquirido de la enfermedad y su epidemiología, incluyendo el papel de las garrapatas (Sanchez-Bojita, 1963) y el diseño de técnicas serológicas para su detección (Canals *et al.*, 1990). Sin embargo, parece que una gran parte de ellos se vio frenado cuando la enfermedad desapareció de Europa (a excepción de Cerdeña) y quedó relegada al continente africano.

3.2.3. Este de Europa

El importante crecimiento del sector porcino en el continente africano, sumado a la absoluta falta de control del movimiento de animales y productos en el continente, y el incremento notable de las comunicaciones y movimientos de personas y productos, han podido propiciar la gran difusión del vPPA en África y su salida a Europa (Costard *et al.*, 2009).

Fue precisamente, en este contexto, cuando se produjo la tercera introducción del vPPA a Europa, esta vez a través de Georgia, en Abril del 2007. El vPPA, en esta ocasión perteneciente al genotipo II (Rowlands *et al.*, 2008), llegó al Este de Europa probablemente a través de comida contaminada transportada en barcos. Una vez allí, entraría en contacto con animales susceptibles en las cercanías del puerto de Poti (Beltran-Alcrudo *et al.*, 2008). Durante los primeros meses de infección, aprovechando la falta de conocimiento y capacidad para implementar las medidas de control adecuadas, el vPPA se difundió rápidamente por el resto del país alcanzando países vecinos como Armenia, Azerbaiyán y, con un impacto mucho mayor, la Federación Rusa. Esta se vio afectada por primera vez en Noviembre 2007 por un foco de jabalí, pero en pocos meses la enfermedad alcanzó al cerdo doméstico, difundiéndose rápidamente en el sur del país.

Figura 15: Mapa representando los focos de PPA declarados a la OIE en el Este de Europa desde 2007 a Abril 2014 (Fuente: OIE-Wahid, elaboración propia).

Desde la zona Sur de Rusia, la PPA fue avanzando poco a poco en dirección norte, hasta que a finales de 2011 se estableció una segunda zona endémica en el Centro-Norte del país (en la región Tver) (Oganesyan *et al.*, 2013). Desde allí el vPPA ha continuado avanzando, ocasionando brotes en 2012 y 2013, en Ucrania y Bielorrusia, respectivamente. Con el objetivo de comprender la evolución de la PPA en Este de Europa, sus efectos negativos y los motivos de la falta de control, se desarrolló un estudio sobre la evolución del vPPA en el Este de Europa recogido en el objetivo 1 de la presente tesis.

A pesar de la ausencia de notificaciones a la OIE, es bastante probable que la PPA permanezca presente en Bielorrusia y Ucrania (EFSA, 2014). Este hecho parece aún más probable desde que en Enero de 2014 se detectase la presencia de dos jabalíes muertos positivos al vPPA en la frontera de Lituania con Bielorrusia (OIE, 2014b). Al poco tiempo, en Febrero 2014, otros dos jabalíes muertos fueron confirmados como positivos al vPPA en Polonia, a tan sólo 900m de la frontera con territorio bielorruso, y en Junio 2014 cuatro jabalíes positivos fueron confirmados en Polonia y uno más en Letonia (OIE, 2014b). La notificación de estos casos positivos en países de la UE ha supuesto consecuencias inesperadas, incluyendo el cierre de exportaciones de toda la UE a la Federación Rusa.

En estos momentos, la PPA vive uno de sus momentos de máxima actualidad, principalmente por la falta de control en el Este de Europa, lo cual supone un riesgo no sólo para la UE, sino para todos los países del mundo. No obstante, no debemos olvidar que la amenaza procedente de África continua presente, ya que el vPPA sigue afectando a un gran número de países en el continente.

3.3.CONSECUENCIAS SOCIO-ECONÓMICAS DE LA PPA

La PPA es una enfermedad de declaración obligatoria a la OIE. Ello conlleva que los países afectados suelen ver seriamente dañado su comercio de exportación de cerdos y productos derivados por las restricciones internacionales impuestas. Además de estas restricciones, que en el caso de países netamente exportadores (como la UE y España) suponen un auténtico desastre, la presencia de la enfermedad ocasiona importantes pérdidas económicas tanto por la muerte de los animales, como por la implementación de las medidas de control.

Por todo ello, a pesar de las diferencias en cuanto a la presentación de la enfermedad y los ciclos epidemiológicos, la PPA se considera una **enfermedad devastadora** para los países que la sufren.

Bien es cierto, que la magnitud y el tipo de estas consecuencias negativas variarán en función del escenario afectado. La importancia del sector porcino en el país afectado, su potencial exportador, así como el tipo de ciclo epidemiológico de PPA establecido en la zona (doméstico, selvático, presencia o no de garrapatas, etc.), determinarán el tipo y magnitud de estas consecuencias.

3.3.1. África

En África la PPA supone el principal freno a la producción porcina en los países sub-subsaharianos (AU-IBAR, 2009). Hasta hace muy poco, la única forma de producción de cerdo en el continente eran los sistemas a pequeña escala, de forma familiar, y en su mayoría con los animales pastando libremente en los alrededores del pueblo/aldea. En muchos casos suponía la base de la economía de subsistencia, proporcionando proteínas, actuando como fuente de ingresos y moneda de cambio.

Por ello, en este continente, la PPA afecta a los más desfavorecidos, a los pequeños productores, que son normalmente incapaces de implementar las medidas de prevención y control adecuadas (Edelsten y Chinombo, 1995). A menudo los productores afectados no tiene recursos suficientes para volver a comenzar con la producción, ya que en el continente africano las compensaciones a los ganaderos son inexistentes en la mayoría de los casos. Esto conlleva que la entrada de la PPA en determinados países (Costa de Marfil (El Hicheri *et al.*, 1998) o Madagascar) haya supuesto la reducción del 30 o 50 % del censo porcino (Roger *et al.*, 2001). Como ejemplo podemos citar la pérdida de 125.000 cerdos en Nigeria en 1997 durante la primera oleada de la enfermedad (El Hicheri *et al.*, 1998), así como los 140.000 cerdos muertos en Chad durante el primer año de aparición de la PPA, en Octubre 2010 (OIE, 2012b).

3.3.2. España

Esto difiere en gran medida con la situación en España, donde la PPA supuso importantes costes al estado, además de una barrera para la exportación de cerdos y sus productos. Estos problemas adquirieron especial importancia cuando España empezó los planes para entrar en el Comunidad Económica Europea (CEE, actualmente UE), para lo cual la PPA suponía una traba para el sector porcino español, que en aquella época ya empezaba a despuntar. Muchos productores se vieron afectados, ocasionando importantes bajas y un importante impacto en el sector. En 1987, tras 27 años de presencia de la enfermedad en el país se habían producido casi 28.000 focos, afectando a aproximadamente 4 millones de animales. Estos supuso unas bajas medias anuales del 39,19% del incremento medio anual del censo porcino, lo que desencadenó un aumento del precio del producto y reducción de la competitividad del sector (Anónimo, 1987).

En 1985 se puso en marcha el programa coordinado para la erradicación de la PPA, que contó con la ayuda económica de la UE (en aquel entonces CEE), con 72 millones de dólares para los primeros cinco años del programa. En algunas zonas de España, donde el cerdo doméstico en intensivo era el tipo de producción mayoritaria, la PPA se controló de forma rápida en los primeros cinco años del programa (Figura 16, a y b). Sin embargo, en otras zonas del país donde la producción era del tipo extensivo, y por lo tanto el cerdo ibérico podía entrar en potencial contacto con garrapatas, otros cerdos y jabalíes infectados, la erradicación se demoró diez años más (Figura 16, c y d). Finalmente, en 1995, gracias a la implantación de un programa coordinado cofinanciado por la UE, la PPA se erradicó de España.

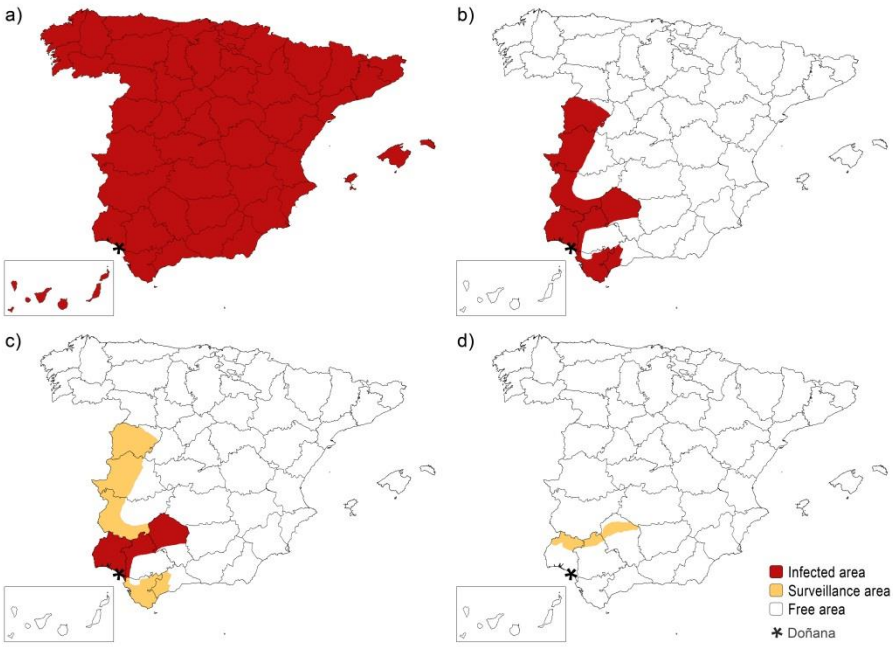

Figura 16: Evolución del programa de erradicación de la PPA en España. a) 1985; b) 1989; c) 1994 y d) 1995. Fuente: (Mur *et al.*, 2012a)

Se estimó un coste medio anual de 30 millones de dólares para estos últimos años del programa de erradicación. Sin embargo, a pesar del elevado coste, podemos asegurar que los esfuerzos invertidos merecieron la pena, ya que la no erradicación de la PPA habría supuesto consecuencias mucho más negativas para España (Bech-Nielsen *et al.*, 1993).

3.3.3. Este de Europa

Una situación relativamente similar es la que se observa actualmente en el Este de Europa, especialmente en Rusia, único país de la zona donde hay acceso a cierta información y nos permite evaluar un poco mejor las consecuencias. Por el contrario, en el resto de países la falta de transparencia respecto a la presencia de la enfermedad y el control de la misma, imposibilitan cualquier tipo de análisis. En Rusia, donde la PPA permanece presente desde noviembre 2007, se han notificado más de 370 focos a la OIE, lo que ha conllevado el sacrificio de aproximadamente 600. 000 cerdos (Dietze *et al.*, 2012). Algunos autores estimaron las pérdidas ocasionadas por la presencia de la PPA, sólo durante el año 2012, en 240 millones de dólares (Callaway, 2012). Por lo que las pérdidas totales ascenderán a más de un billón de dólares americanos para todo el periodo.

A pesar de ello, Rusia presenta un situación favorable, ya que es un país principalmente importador, por lo que el cierre de exportaciones no ha supuesto un problema para el país. De

hecho, a pesar de los problemas ocasionado por la PPA, Rusia ha aumentado de forma notable su producción porcina, alcanzando los 2,6 millones de toneladas producidas. El gobierno ruso está favoreciendo la modernización e industrialización de las granjas, en detrimento de los pequeños productores, considerados como un riesgo para la difusión y permanencia de la PPA en el país (USDA, 2014).

Esta situación sin embargo es diametralmente opuesta a la de la Unión Europea, gran exportador de carne de cerdo (2,2 millones de toneladas exportadas en 2012), cuyo principal mercado está en Asia y países del Este. De hecho, hasta hace muy poco, Rusia era el primer destino de estas exportaciones, que en el año 2013 ascendieron a 1.400 millones de euros (25% del total de carne exportada desde la UE) (EC, 2013). Sin embargo, tras la notificación de los casos de jabalíes muertos observados en las fronteras de Polonia y Lituania, Rusia decidió cerrar el mercado a las importaciones de cerdo y cualquier tipo de producto procedente de todos los países de la UE. Esta medida ha supuesto un importante revés para la UE, provocando la disminución del precio del producto y el exceso de oferta interna en la UE. Ante esta considerada totalmente desmesurada e injustificada, la UE ha reclamado ante la Organización Mundial de Comercio (OMC) (CE, 2014). A la espera de una solución, se han abierto otros mercados como Japón y el resto de Asia con el objetivo de dar salida a la producción de cerdo excedente y evitar así las pérdidas ocasionadas.

Por lo tanto, las consecuencias de la PPA variarán en función de las características del país afectado, pudiendo alcanzar cuotas difíciles de prever, debido a las pérdidas indirectas ocasionadas. Estas incluyen tanto el impacto en el comercio internacional, como por el incremento de los costes de producción, el encarecimiento del producto y otras pérdidas ocasionadas de forma indirecta por la presencia de la enfermedad.

3.4.HERRAMIENTAS DISPONIBLES PARA LA PREVENCIÓN Y CONTROL DE LA PPA

3.4.1. Prevención

Las importantes consecuencias negativas de la entrada de PPA hacen vital evitar la introducción del vPPA en los países libres. La falta de vacuna limita las posibilidades existentes a las medidas clásicas y estándar aplicadas para la prevención de cualquier enfermedad animal. Sin embargo, cabe destacar que la PPA no se transmite a largas distancias ni por aerosoles, sino es con

la ayuda del ser humano, por lo que las medidas de prevención en países libres incluirán controles estrictos y aplicación de bioseguridad adecuada (Penrith y Vosloo, 2009, Arias *et al.*, 2002).

Control de entrada de productos contaminados: El Código Terrestre de la OIE (OIE, 2011) proporciona una serie de guías para garantizar la importación de cerdos y productos derivados del cerdo de forma segura. Esta información deberá ser complementada con un conocimiento adecuado de la situación epidemiológica actual para conocer la distribución y el origen del riesgo. Además, en el caso de animales importados, será de gran importancia realizar una fase de **cuarentena**, en las instalaciones adecuadas para este uso, con el fin de observar la aparición de enfermedad antes de su introducción en granja.

Sin embargo, la vía de entrada más común de PPA a países libres a lo largo de los años han sido los restos de comida contaminados (restos de catering) traídos a partir de barcos y aviones internacionales. Por ello, es altamente recomendable la incineración de todos los restos de comida procedentes de aviones y barcos internacionales (Sánchez-Vizcaíno y Arias, 2012). En las zonas donde esta medida no es viable (véase África), será recomendable el tratamiento de los productos previo empleo para alimentación animal, o limitar el acceso de los cerdos mediante el vallado adecuado de los recintos.

También puede darse la entrada de productos contaminados por importaciones ilegales, tanto para uso personal como fines comerciales (Wooldridge *et al.*, 2006). Por lo que se deberá intensificar la vigilancia en aeropuertos, puertos y fronteras para evitar la entrada de alimentos y residuos procedentes de zonas contaminadas (Sánchez-Botija, 1982).

Prohibición de alimentación con desperdicios: Estos productos contaminados no conllevarían ningún problema si no fuese porque en un momento dado son proporcionados a los cerdos como alimento. Por ello, una medida de prevención fundamental en el caso de la PPA es prohibir la alimentación a base de desperdicios, especialmente si estos contienen productos derivados del cerdo. Esta es una práctica de alto riego prohibida en la UE desde 2002, a raíz de los brotes de Fiebre aftosa ocurridos en el año 2001 (EC, 2002). Sin embargo, de forma no oficial continúa estando presente en ciertas regiones y sistemas de producción, con medidas de bioseguridad más limitadas.

Bioseguridad: El incremento de los niveles de bioseguridad de las explotaciones porcinas reduce de forma considerable el riesgo de entrada de enfermedades. La existencia de unos protocolos adecuados de cuarentena, control de visitas, vallados, control de la alimentación, agua y otras fuentes externas, así como adecuadas instalaciones de carga y descarga, dificultan la entrada de enfermedades. Estos factores, extremadamente importantes, deberán reforzarse en las zonas consideradas de alto riesgo, donde se debe evitar además el contacto de los animales con la población silvestre. Para ello es aconsejable el confinamiento de los animales en las instalaciones, evitando prácticas en extensivo, reformando el vallado perimetral de la instalación.

Campañas de información que difundan el riesgo existente y pongan a la población, especialmente veterinarios y ganaderos, en alerta sobre la enfermedad. Se deberá incluir información sobre la forma de presentación de la enfermedad, lesiones típicas y cómo actuar en caso de sospecha (Beltran Alcrudo *et al.*, 2009).

Análisis de riesgo: Esta herramienta ha sido señalada con anterioridad por otros autores como de gran utilidad para la prevención de la entrada de PPA en países libres (Costard *et al.*, 2009). Su implementación permitiría a los países en los que la producción porcina es importante, conocer cuáles son las vías y regiones con mayor riesgo para la introducción, y actuar en consecuencia.

Algunos análisis de riesgo han sido realizados para la estimación del riesgo de entrada de PPA en determinados territorios. Si bien, hasta la fecha todos ellos se limitaban a estudiar el riesgo en una única unidad de análisis (un país en su conjunto), sin diferenciar entre las regiones/ estados. En el objetivo 2 de la presente tesis se incluyeron tres estudios de análisis de riesgo sobre la introducción de PPA en la UE por medio de dos vías distintas. A su vez, se desarrolló un modelo para la integración del análisis de riesgo de cinco vías diferentes de entrada para los 27 países de la UE, lo que proporciona una información global del riesgo, de gran importancia para evitar la entrada de PPA.

3.4.2. Métodos de control

La PPA es una enfermedad vírica transmisible para la cual no existe en la actualidad tratamiento ni vacuna disponible.

Se han realizado ciertos estudios respecto a la utilidad de antivirales para frenar los síntomas y evolución de la infección del vPPA. Sin embargo, los resultados obtenidos hasta el momento no han sido del todo esperanzadores (Tignon *et al.*, 2012).

Al tratarse de una enfermedad de declaración obligatoria a la OIE, es fundamental una **detección y diagnóstico confirmatorio rápido** que permita implementar las medidas de control de forma inmediata. No obstante, antes incluso de obtener el resultado oficial del laboratorio, ante una sospecha de PPA, se deberán poner en marcha unas medidas cautelares que eviten la propagación del virus a otras explotaciones.

En el caso de España, al igual que en el resto de países de la UE, existe una normativa de obligado cumplimiento que regula las medidas de control, su duración y forma de implementación en el caso de sospecha y confirmación de PPA (EC, 2002). De forma general, el control de PPA implica la eliminación y destrucción de los animales afectados, y el control exhaustivo de movimientos. Los puntos fundamentales del control de PPA se desarrollan a continuación:

Declaración obligatoria de la enfermedad, tanto de las formas clínicas observadas, como de las posibles sospechas.

Secuestro e inmovilización inmediata de la explotación afectada y las vecinas. Para evitar la aparición de prácticas ilegales como el esconder animales o la venta apresurada previa a la llegada del veterinario oficial, es fundamental realizar **un censo detallado** de todos los efectivos presentes en granja en el primer momento de la sospecha.

Sacrificio obligatorio y eliminación apropiada de los animales infectados. Todo brote de PPA deberá ser eliminado de forma rápida, mediante el sacrificio de todos los animales presentes en la explotación, así como los que han podido tener contacto con ellos. Se tomarán **muestras** de los animales para confirmar la presencia de la enfermedad mediante diagnóstico laboratorial.

Las canales de estos animales deben ser correctamente **eliminadas**, de forma que se evite cualquier contacto con las mismas. Para ello se trasladarán a unas plantas especiales de procesamiento en vehículos sellados, o serán quemadas y/o enterradas in situ. Tras el sacrificio se deberá llevar a cabo una **limpieza y desinfección** en profundidad que asegure la eliminación de cualquier resto de material contaminado. Se emplearán los productos adecuados, siguiendo los protocolos establecidos por las autoridades competentes.

Desde el momento de la primera sospecha, se deberá establecer un **estricto control de movimientos**, prohibiendo la salida de cualquier animal y producto de la explotación afectada. Del mismo modo, se establecerán unos radios de 3 (zona de control) y 10 km (zona de vigilancia) alrededor de la explotación, en los cuales se prohibirá el movimiento de cerdos. Además en las explotaciones localizadas dentro de estos radios se deberán realizar controles clínicos y serológicos (en el caso de la zona de control). Dentro de estas zonas también se prohibirá la realización de ferias y mercados de animales.

En las zonas en las que se sospeche de la presencia e intervención de **las garrapatas** en la aparición del foco, se deberán llevar a cabo las medidas adecuadas de control. Lo mismo ocurre con los jabalíes, frente a los cuales se deberán mejorar las infraestructuras y vallado de la explotación, de tal forma que garantice el aislamiento de los cerdos con la **población silvestre**.

Estas son las medidas generales a aplicar en presencia de un foco de PPA. Sin embargo, estas medidas deberían adaptarse a la región afectada, el escenario epidemiológico, los recursos económicos disponibles y la situación de los países vecinos (Sánchez-Vizcaíno y Arias, 2012).

Con el paso de los años, en España se fueron desarrollando nuevas estrategias para el control de la PPA, basadas en la experiencia obtenida de los años previos (Sánchez-Botija, 1982). Para el control de los focos se observó que la participación activa del ganadero era fundamental en el proceso. Así que además de implementar un adecuado **programa de compensaciones**, que recompensase al ganadero por los daños sufridos, se crearon asociaciones de ganaderos. Estas Agrupaciones de Defensa Sanitaria (ADS) recibían privilegios por parte del Gobierno, consiguiendo a cambio una mejora sustancial de la calidad sanitaria del sector. Del mismo modo, se fueron promocionando las ayudas para la mejora de las instalaciones y la instauración de medidas biosanitarias adecuadas, así como para la construcción de nuevas instalaciones de ciclo cerrado industrial (Sánchez-Botija, 1982) .

Estos conocimientos fueron aplicados en el desarrollo del **programa coordinado para la erradicación de PPA en España**, artífice del éxito de la erradicación en 1995. Los pilares principales de este programa fueron (Arias y Sánchez-Vizcaíno, 2002)

i) el desarrollo de un equipo de veterinarios móviles responsables de la aplicación directa del programa;

ii) el control serológico del 100% de la población mediante la técnica innovadora por aquel entonces del ELISA;

iii) mejora de las infraestructuras de las explotaciones de porcina

iv) eliminación de todos los brotes y animales portadores detectados

v) identificación animal y control exhaustivo de movimientos de animales

Estas fueron las claves para lograr la erradicación en España. Sin embargo, no existe una receta única para acabar con la enfermedad, y por lo tanto, estas no tienen por qué ser las medidas adecuadas para otros países. En otros territorios afectados como Cuba, tras el rebrote en 1980, se establecieron medidas adicionales a las tradicionales anteriormente comentadas. Un ejemplo son los 229 puntos de control establecidos en el país para controlar la entrada y/o salida de ciudades y localizaciones con riesgo. Estos puntos, que operaban 24 horas al día, controlaban tanto los equipajes como mercancías de viajeros, camiones, etc. Además, al igual que ocurrió en España, las campañas de publicidad e información en los medios fueron masivas tanto en prensa escrita como en radio con el fin de informar a la población (Simeon-Negrin y Frias-Lepoureau, 2002).

En el objetivo tres se desarrollaron metodologías para el análisis de los factores de riesgo implicados en zonas endémicas, que permitiesen la adaptación de los planes de control de PPA a las características específicas de la zona. Para ello, se tomó Cerdeña como escenario modelo, así como España para el estudio del papel de jabalí en el mantenimiento de la enfermedad.

4. JUSTIFICACIÓN Y OBJETIVOS

Fuente: imagen propia

4. JUSTIFICACIÓN Y OBJETIVOS

La PPA es una enfermedad de declaración obligatoria del ganado porcino cuya presencia implica graves consecuencias socio-económicas en los países que la padecen, incluyendo un gran número de países de África subsahariana y la isla de Cerdeña. Sin embargo, el gran cambio en la epidemiología de la enfermedad ocurrió en 2007 cuando la PPA se introdujo en Georgia. Desde entonces, la enfermedad se ha difundido en los países vecinos y a día de hoy se ha establecido en el Este de Europa, llegando incluso recientemente a alcanzar las fronteras de la UE. Esto ha despertado todas las alarmas y preocupación de la industria porcina mundial, y en especial la europea, ante las graves consecuencias que una posible entrada de PPA podría ocasionar.

Objetivo general

En esta tesis se han desarrollado herramientas y estrategias que permitirán evitar la entrada de la PPA en los países libres mejorando los planes de vigilancia y detección temprana; así como ayudar al control y erradicación de la enfermedad en países afectados e incluso endémicos.

Los ***objetivos concretos*** de esta tesis son:

1) Evaluación epidemiológica de la situación de la PPA e identificación de los factores de riesgo presentes en los distintos escenarios afectados, con especial atención al Este de Europa.
2) Caracterización del riesgo de introducción del vPPA en la UE para dos de las vías de entrada más importantes (comercio legal de cerdos y riesgos asociados al transporte) y desarrollo de una herramienta modular para la integración de análisis de riesgo.
3) Estudio de los factores de riesgo que dificultan el control de la PPA en zonas endémicas, para facilitar el diseño y adaptación de planes de contingencia y erradicación basados en riesgo más eficaces y con mejor coste-beneficio.
4) Desarrollo de nuevas metodologías diagnósticas para la vigilancia de PPA en campo, concretamente, mediante la adaptación de técnicas serológicas para el diagnóstico de PPA en fluido oral.

5. PUBLICACIONES/PUBLICATIONS

Fuente: imagen propia

OBJETIVO 1: Situación epidemiológica actual de la PPA e identificación de los factores que dificultan su control en el Este de Europa

La incidencia de PPA no ha aumentado únicamente en el continente africano desde los últimos 15 años, afectando al Oeste de África, Mauricio y Madagascar, sino que también ha alcanzado nuevos territorios como la región del Cáucaso en 2007. Desde que la PPA fue reintroducida en el Este de Europa en Abril de 2007, la enfermedad se ha difundido por los países vecinos cambiando de forma drástica la situación de la PPA en Europa. Esta re-introducción tiene importantes consecuencias para los países afectados y pone a la UE en serio riesgo para la introducción de PPA.

Se realizó una evaluación de la situación actual de la PPA y sus distintos ciclos epidemiológicos, con especial interés en el Este de Europa, incluyendo una identificación de los factores que pueden contribuir a la endemicidad en esta área y suponen un reto importante para el control de la enfermedad en la zona. En base a estos, se destacó la necesidad del desarrollo y promoción de esfuerzos globales para la prevención y control de la PPA.

Se identificaron numerosos factores presentes en Rusia y zonas adyacentes, que complican el control de la PPA en la zona. Destacan especialmente, la ausencia de un programa coordinada de control, la abundancia de producción de traspatio, o el uso de restos de comida para la alimentación de los animales. Siendo todos estos factores de riesgo de tipo socio-económico, político y cultural. Además la falta de información transparente respecto a la situación de la PPA en los países transcaucásicos como Armenia y Georgia supone un riesgo importante para la difusión de PPA a áreas vecinas.

Los resultados de estos análisis nos permitieron identificar la problemática de PPA en el Este de Europa, y en base a ella, proponer estrategias y soluciones que se desarrollaron en los siguientes objetivos de la tesis, que permitiesen prevenir la entrada de la PPA en la UE y controlar su difusión en los territorios endémicos.

Artículos científicos:

- Incluidos en la tesis:
    - Sánchez-Vizcaíno JM., Mur L., Martínez-López B., 2012: **African Swine Fever: An Epidemiological Update**. *Transb Emerg Dis*. 59, 27-35.

- Sánchez-Vizcaíno JM., Mur L., Martínez-López B., 2013: **African swine fever (ASF): Five years around Europe.** *Vet Microbiol*. 165, 45-50.

- No incluidos en la tesis:
  - Costard S., Mur L., Lubroth J., Sanchez-Vizcaino JM., Pfeiffer DU., 2013. **Epidemiology of African Swine Fever Virus.** *Virus Res*. 173, 191-197.

ORIGINAL ARTICLE

African Swine Fever: An Epidemiological Update

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Summary

African swine fever (ASF) is one of the most important swine diseases, mainly because of its significant sanitary and socioeconomic consequences. This review gives an update on the epidemiology of the disease and reviews key issues and strategies to improve control of the disease and promote its eradication. Several characteristics of ASF virus (ASFV) make its control and eradication difficult, including the absence of available vaccines, marked virus resistance in infected material and contaminated animal products, and a complex epidemiology and transmission involving tick reservoir virus interactions. The incidence of ASF has not only increased on the African continent over the last 15 years, so that it now affects West African countries, Mauritius and Madagascar, but it has also reached new areas, such as the Caucasus region in 2007. In fact, the rapid spread of the disease on the European continent and the uncontrolled situation in the Russian Federation places all countries at great risk as a result of intense global trade. The proximity of some affected areas to the European Union (EU) borders (<150 km) has increased concerns about the potential economic consequences of an ASF incursion into the EU pig sector. Establishing effective surveillance, control and eradication programmes that implicate all actors (veterinarians, farmers, and policy makers) is essential for controlling ASF. African swine fever -free countries should be aware of the potential risk of ASF incursion and implement risk reduction measures such as trade controls and other sanitary measures. This review will discuss lessons learnt so far about ASF control, current challenges to its control and future studies needed to support global efforts at prevention and control.

Introduction

African swine fever (ASF) was first described by Montgomery in Kenya in 1921. Since then, many African, European and American countries have been affected by the disease. African swine fever is one of the most complex and economically devastating viral diseases in swine herds, producing great socioeconomic impact in affected countries. For that reason, it is listed as a notifiable disease by the World Organization for Animal Health (OIE). African swine fever has several properties that make it difficult to control and eradicate, which are outlined below.

African swine fever virus (ASFV) is a very complex and large enveloped DNA virus with a genome of 170–190 kbp, and it is classified as a unique member of the *Asfarviridae* family, genus *Asfivirus* (Dixon et al., 2005). The virus presents high genetic and antigenic variability, with 22 different genotypes described based on the p72 sequences, all of which currently circulate on the African continent (Boshoff et al., 2007).

The natural hosts of ASFV are African wild pigs; however, wild boars and domestic pigs of all breeds and ages are also susceptible to the infection by ASFV. The virus also infects different species of soft ticks of the genus *Ornithodoros*, in which it can persist more than 5 years (Oleaga-Pérez et al., 1990). In its natural hosts, ASFV targets primarily monocytes and macrophages of the mononuclear phagocytic system (Malmquist and Hay, 1960).

Protection against ASFV is not fully understood. Although ASFV infection induces small proportion of neutralizing antibodies against some virion proteins (Ruiz-Gonzalvo et al., 1986; Zsak et al., 1993; Gomez-Puertas et al., 1996), this protection is not enough for viral challenge (Neilan et al., 2004). Despite that, role of antibodies is important, as they confer some protection that does reduce viraemia, delay the onset of clinical signs and reduce the adverse effects of infection, as it was demonstrated by passive transferred of anti-ASFV immunoglobulins by Onisk et al., 1994. Cellular immunity also plays an important role in immune protection against ASFV infection, specifically, cell activity of CD8 lymphocytes (Oura et al., 2005) and natural killer cells (NK) (Leitão et al., 2001). Cross-protection has been also demonstrated by challenging infected animals with homologous isolates, (Mebus and Dardiri, 1980; Ruiz-Gonzalvo et al., 1986), some of them field low virulent strains (Leitão et al., 2001; Boinas et al., 2004; King et al., 2011).

Nevertheless, all the previous attempts to develop a vaccine have failed and no effective vaccine is available against ASF. Live attenuated vaccines were previously used in Spain and Portugal, but their use implies unacceptable safety problems (Manso Ribeiro et al., 1963). Indeed, the vaccination applied in Iberian Peninsula with these live attenuated vaccines during 1960s might have been the origin of some low virulence strains that induced the onset of chronic forms of the disease during ASF infection in the Iberian Peninsula (1960–1995).

Other strategies such as the use of deleted replicating viruses with one or more, naturally or experimentally, deleted genes (Alfonso et al., 1998; Lewis et al., 2000; Salguero et al., 2008), subunit vaccines based on recombinant proteins (Ruiz-Gonzalvo et al., 1996; Gomez-Puertas et al., 1998; Neilan et al., 2004; Ivanov et al., 2011) or DNA vaccines (Perez-Martin et al., 2006; Argilaguet et al., 2011; Lacasta et al., 2011), did no confer a complete real protection.

In summary, all the studies on ASF protection until now only proved the delay of the onset of clinical signs and death and partial protection against homologous viruses; but none of them has the adequate and required properties to be an effective and safe vaccine against ASF.

African swine fever virus is highly resistant to inactivation in the environment in the presence of organic material. The virus persists for long periods of time in infected material (blood, faeces, serum, slurry) and tissues, where it can survive for more than 15 weeks in putrefied blood, 11 days in faeces kept at room temperature or 1000 days in frozen meat (European Food Safety Authority (EFSA), 2009). This great persistence must be considered when developing ASF contingency and eradication plans, so that appropriate disinfectants and procedures should be used to achieve complete disinfection of contaminated areas and materials.

African swine fever is considered a haemorrhagic disease due to the typical haemorrhagic symptoms of the hyperacute and acute forms of the diseases, although other presentations (chronic and asymptomatic forms) of the disease are shown without these characteristic symptoms. African swine fever progresses with different clinical signs depending on isolate virulence, host, dose and route of exposure. Hyperacute and acute clinical signs in pigs and wild boars are very similar to those of other haemorrhagic diseases, such as classical swine fever, salmonellosis or erysipelas. Thus, laboratory diagnosis is required for differentiating among them. African swine fever clinical signs may vary from a hyperacute form, with 100% mortality from days 4–7 post-infection and typical haemorrhagic symptoms, to a less common asymptomatic and chronic form that can turn animals into carriers. This last form, observed in wild and domestic animals, plays an important role in the persistence and dissemination of the disease in endemic areas, even more if infected ticks are present (Arias and Sánchez-Vizcaíno, 2002a).

European wild boars are usually more resistant than domestic pigs to ASFV infection, although they present a similar pathological and epidemiological pattern (Sánchez-Vizcaíno, 2006). African wild suids such as warthogs (genus *Phacochoerus*), bush pigs (*Potamochoerus porcus* and *P. larvatus*) and giant forest hogs (*Hylochoerus meinertzhageni*) may also be infected by ASFV, but they usually do not exhibit clinical signs, allowing them to act as reservoir hosts in Africa (De Tray, 1957). In these wild suids, ASF infection is characterized by low levels of virus in tissues and low or undetectable viraemia (Plowright, 1981). These levels of virus in adult suids are insufficient for transmission through direct contact between animals and/or indirect contact by ticks (Jori and Bastos, 2009). However, ASF transmission occurs repeatedly in warthog burrows, between infected soft ticks and neonatal warthogs that develop high levels of viraemia, sufficient to infect naive ticks that feed on them (Thomson, 1985). The role of these wild suids, especially warthogs, in the sylvatic cycle of ASF in Africa, is essential for the persistence of the disease in the continent, creating serious difficulties for the eradication of the disease in eastern and southern Africa. However, in West African countries, the role of wild suids and ticks seems to be less important for ASF spreading and endemicity (Penrith and Vosloo, 2009). In these countries, the disease is maintained and spread mainly by movements and illegal trade of infected pigs and pork products.

Depending on the existence of wild reservoirs and ticks, and their interactions with domestic suids, five ASF epidemiological scenarios can occur. The most ancient

scenario describes transmission from eastern and southern African countries; this involves a sylvatic cycle explained before, where wild suids and soft ticks from the *O. moubata* species act as ASFV reservoirs. African swine fever transmission to domestic pigs is mainly caused by the bites of infected ticks or by the ingestion of tissues from acute-infected warthogs (Wilkinson, 1986).

The second scenario describes the situation in West African countries, which have been more recently affected by ASF. Here, transmission occurs mainly through direct contact between domestic pigs or indirect contact between pigs and pork products, but no soft ticks are apparently involved. Socioeconomic factors such as lack of veterinary services, lack of compensation to farmers for culled animals and the consequent hiding of the diseased animals in its first stages, facilitate the spread of the disease within the country and to neighbouring countries. This situation is also occurring in some areas of the Caucasus and the Russian Federation (Beltrán-Alcrudo et al., 2009).

A third scenario occurred when the disease was present on the Iberian Peninsula (1960–1995). Domestic pigs and wild boars suffered the disease, which was mainly transmitted by direct contact between animals and infected meat. *O. erraticus* soft ticks also contribute to disease transmission in outdoor pig production systems (Arias and Sánchez-Vizcaíno, 2002b), as well as reservoir of ASFV in previous infected areas, in which, these ticks are able to transmit the disease 1 year after the removal of the infectious host, and allow the persistence of ASFV for 5 years (Boinas et al., 2011). However, in contrast to *O. moubata*, *O. erraticus* ticks participate only in transtadial ASFV transmission, so no transovarial transmission has been observed (Plowright, 1981). In this scenario, the presence of infected wild boars and these soft ticks made ASFV eradication difficult, particularly in outdoor swine production areas, where *O. erraticus* was the cause of re-emergences of the disease, even after disease eradication, as it was the case of the single outbreak in Portugal in 1999 (Boinas et al., 2011). Nevertheless, after tremendous efforts, eradication was achieved through programmes that included the detection of anti-tick antibodies in domestic and wild boars, as well as the destruction or isolation of the pigpens where ticks were present (Arias and Sánchez-Vizcaíno, 2002b). Another model of this scenario occurs in certain areas of Central Africa (demonstrated in Malawi (Haresnape et al., 1988), and probably neighbouring Mozambique and Zambia), where ASF is maintained within the domestic pig population, that could also get infected by soft ticks present in the pig pens.

A similar situation occurred in the fourth scenario, when the disease was introduced into Central and South American countries (1968–1980), but this time the disease affected only domestic pigs, as wild animals (feral pigs) and soft ticks did not play an important role in the epidemiology and transmission of the disease. The absence of wild reservoirs or ticks facilitated early eradication of the disease after costly eradication campaigns in the area (Simeón-Negrín and Frías-Lepoureau, 2002; Lyra, 2006).

The last scenario occurs in currently affect areas of the Russian Federation and trans-Caucasian countries. The epidemiological cycle of ASF is affecting domestic pigs and wild boars, but so far, ticks are not involved. Most of the outbreaks (79.8%) affect domestic pigs and have been caused by movements of infected or carrier animals and their products. Only 20.2% of outbreaks affect wild boars; these have been mainly caused by contact between wild boars and domestic pigs, whilst transmission within wild boar population also occurs (World Organization for Animal Health, 2011a; European Food Safety Authority (EFSA), 2010a). Although no soft ticks implicated in the cycle have presently been found in the area, some *Ornithodoros* species inhabit the region (European Food Safety Authority (EFSA), 2010a) and all *Ornithodoros* species tested to date are susceptible to ASFV infection (European Food Safety Authority (EFSA), 2010b). For this reason, further studies should be carried out to understand the transmission role of soft ticks in this area.

Understanding the different epidemiological scenarios and the characteristics of the disease is critical for developing successful contingency and eradication plans in affected areas.

ASF Distribution

Since the first description of the disease in Kenya in 1921, many sub-Saharan countries have been affected by ASF. The disease was restricted to this region until 1957, when an outbreak occurred in Portugal, the first outbreak outside the African continent. This outbreak was effectively controlled and eradicated, but new ones occurred in 1960 near Lisbon (Manso Ribeiro and Azevedo, 1961). Recent genetic studies (Gallardo et al., 2009a,b) discriminate between these isolates, and suggest the possibility of two different introductions in the area. From this initial outbreaks, ASFV spread to many areas of the Iberian Peninsula (Spain and Portugal), where it remained endemic until 1995. During the 1970s and 1980s, ASFV travelled around the world, affecting more countries in Europe, such as the Netherlands, Italy, France and Belgium, as well as some countries in the Americas, notably the Dominican Republic and Brazil (Costard et al., 2009). The historical way of ASFV introduction into these disease-free areas was mainly by feeding domestic animals with contaminated pork products that entered the territory via international airports and seaports. Once established in domestic

herds, infected pigs and pork products become the primary sources of virus dissemination. Based on epidemiological data from Spanish scenario, role of carriers in virus dissemination seems to be not so important when appropriate control measures are put in place (Bech-Nielsen et al., 1995). As a result of significant control efforts, the disease was eradicated from all these territories, but it persisted on the Italian island of Sardinia and on the African continent, especially in southeast Africa.

During the 1990s and 2000s, the epidemiology and distribution of the disease changed (Fig. 1): ASFV spread to other regions not typically affected by ASF. These included West African countries, where the virus was first reported in Côte d'Ivoire (1996), Nigeria (1997), Togo (1997), Ghana (1999), Burkina Faso (2003) and recently, Chad (2010). It also spread to some islands, such as Madagascar (1998) and Mauritius (2007). Importantly, the disease re-entered the European continent in 2007, this time via Georgia (World Organization for Animal Health, 2011a).

This considerable epidemiological change may have been caused by a combination of several factors, the most important of which may be the increasing presence of ASFV on the African continent over the last 15 years, as the incidence of ASF has increased in endemic countries and entered previously disease-free territories. This suggests that the amount of virus circulating in the world has increased, together with the number of infected animals and the amount of contaminated pig products. A second important factor is globalization. Nowadays, people, animals and products travel long distances around the world in very short periods of time. This movement increases the potential for introducing pathogens into new territories. The third important factor is the global financial crisis, which has forced small farmers to meet their needs in new ways, such as by using swill or garbage to feed their animals. These three factors, together with the high resistance of ASFV in the environment and meat products, the presence of asymptomatic carrier animals and the lack of vaccine may explain how the disease has not only increased in endemic areas but also spread to new territories.

ASF Situation in the Caucasus Region and the Russian Federation

In April 2007, a new outbreak of ASF p72 genotype II, compatible with the virus circulating in Mozambique, Madagascar and Zambia, reached the European continent via Georgia (Rowlands et al., 2008). Since then, all the

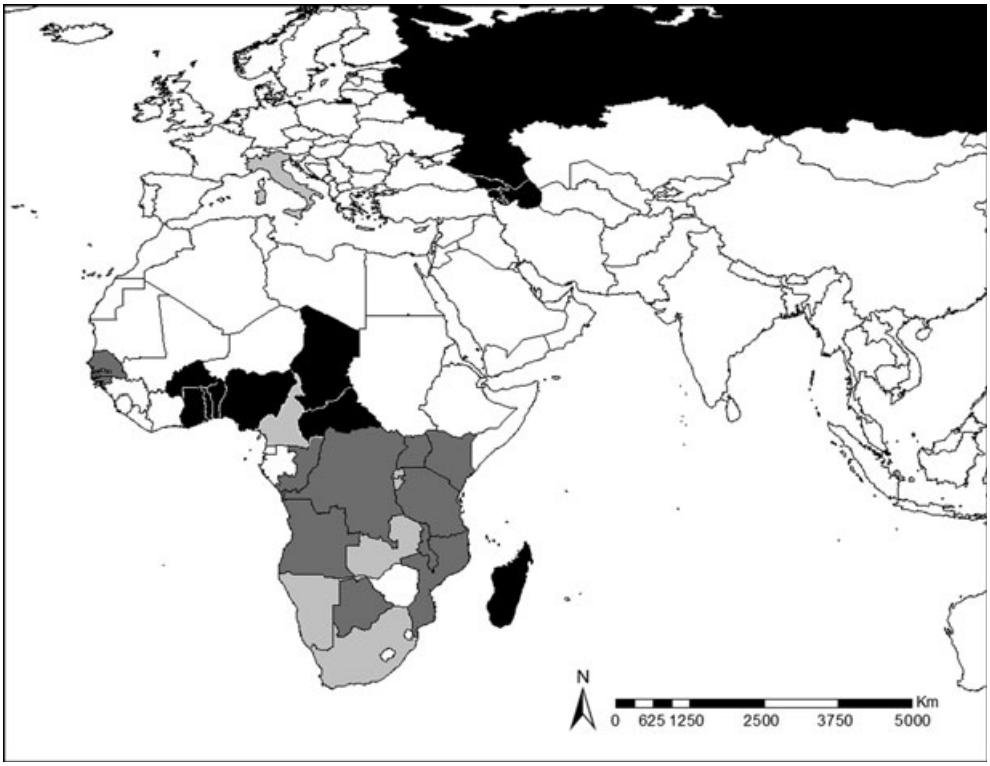

Fig. 1. Countries affected by African swine fever. Light grey indicates countries where disease is limited to a few areas; medium grey, countries where infections occurred before 1995; and black, countries where infections occurred after 1995.

ASFV isolates found in the Caucasus region and Russian Federation show identical p72, p54 and CVR sequences, suggesting that only one virus arrived in the area in 2007 and subsequently spread from this initial outbreak (Gallardo et al., 2009a,b). This ASFV is thought to have been introduced by international ships containing infected swill that were used to feed pigs near the port of Poti (Beltran-Alcrudo et al., 2008). After this introduction, the disease spread very quickly, affecting four different countries: Georgia, Armenia, Azerbaijan and the Russian Federation. Since the introduction of the virus in the Caucasus region, the OIE has been notified of more than 273 outbreaks, in which more of 76 000 animals have died (World Organization for Animal Health, 2011a). The economic losses of these outbreaks in the Russian Federation have been estimated at 25–30 billion RuR (0.8–1 billion USD) (United States Department of Agriculture (USDA), 2010). The likelihood that ASF will become endemic and spread to nearby unaffected areas of the Russian Federation has been estimated as very high (European Food Safety Authority (EFSA), 2010a) due to the presence of some adverse factors in the area: demonstrated ASFV infection in wild boar populations, an extremely high volume of illegal trade of pigs and pork products within the country, a tradition of swill feeding, the absence of adequate veterinary services and lack of pig production infrastructure and traceability (Beltrán-Alcrudo et al., 2009).

However, in some of the affected areas of Russian Federation, like Krasnodar region, important control measures are being applied. These measures are based on early detection and notification of the disease, promoted by education and importantly, compensation to the farmers (Shevkoplyas, 2011). Since the first introduction of the disease in the territory, ASF has been present in a hyperacute–acute form of the disease, without chronic forms of the disease, and an average incubation period of 4.3 days. No changes in the pathological and epidemiological pattern of the disease have been observed by Russian veterinaries that support the absence of recovered or undetected infected animals in their country (Blagodarsnosti, 2011). However, no active surveillance and serological diagnosis are being carried out by official veterinaries in Russian Federation until now.

All the factors mentioned before and the lack of a coordinated national control programme make control and eradication of the disease from this area very difficult, and increase the risk of spread to neighbouring countries, especially those with commercial and sociocultural relationships with the Russian Federation. These observations are supported by recent comments of the Russian Chief Veterinary Officer, who predicted a spread of the disease towards the northern and northwestern regions (Vlasov, 2011). Recent ASF outbreaks in October 2009, December 2010 and April 2011 occurred very near EU borders, less than 150 km away from Estonia, Finland and Norway (Fig. 2) (World Organization for Animal Health, 2009a; World Organization for Animal Health, 2010a; World Organization for Animal Health, 2011b). These outbreaks increase the risk of introduction into the EU.

The EU is aware of the potential risk of ASFV introduction within its borders. A recent risk assessment (European Food Safety Authority (EFSA), 2010a,b; Wieland et al., 2011) estimates the risk of ASFV introduction into the EU as moderate. However, given the spread and uncontrolled situation of the disease in Russia and the recent outbreaks near the EU border, this estimation should be reconsidered. The same risk assessment highlights the risk of introduction through contaminated products used for swill feeding. Historically, this was the most frequent route of ASFV introduction into disease-free countries, e.g. Spain, Netherlands, Belgium, Cuba and more recently, Georgia. At the same time, the risk assessment predicts that once the disease enters the EU, it is unlikely to persist there, given the relatively high biosecurity of the pork production industry.

Although imports of pigs and pig products from ASF-affected areas into the EU have been completely banned since the first notification of the disease (World Organization for Animal Health, 2009b), the EU is aware of the continuing risk, and recently approved a new decision regulating livestock vehicles coming from Russia (Decision 2011/78/EU). As a result, vehicles used for pig transport that enter the EU from Russian territory must certify that they were adequately disinfected after the last uploading of pigs.

More detailed and complete analyses are being developed within the European project ASFRISK (EC, FP7-KBBE-2007-1, Project #211691) to estimate the most likely pathways, countries and months of ASFV introduction into the EU. Preliminary results of this analysis consider the likelihood that ASFV will be introduced into the EU by import of live pigs as low (Mur et al., 2011). Methods and results obtained by this risk assessment may help allocate financial and human resources to areas and periods at higher risk, helping to reduce the risk of ASFV introduction into the EU.

Prevention and Control

Preventing the virus from entering disease-free areas is crucial and must focus on avoiding the introduction of potentially infected pigs or pork products and properly disposing of fomites, particularly pork waste from aircrafts and ships. Once the disease has entered a territory, control measures should aim for early detection in the

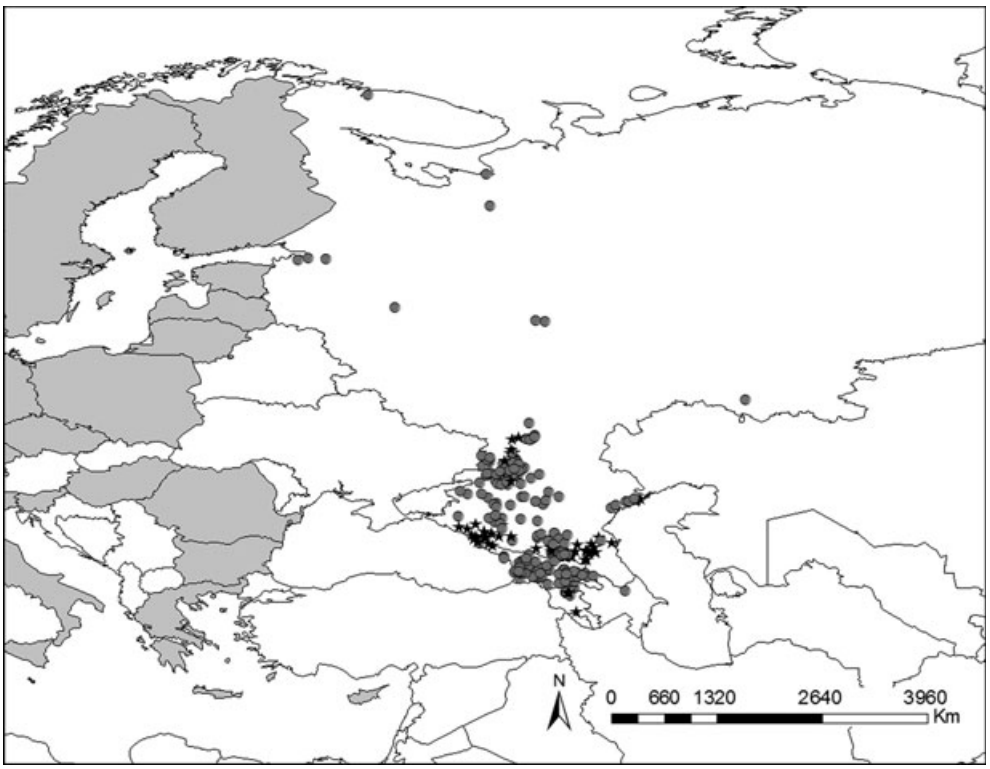

Fig. 2. Outbreaks of African swine fever in domestic pigs (•) and wild boar (★) in the Russian Federation and Trans-Caucasian countries. European Union countries are shown in grey.

field following by a rapid laboratory diagnosis, as well as enforcement of strict sanitary measures (Sánchez-Vizcaíno, 2006). Laboratory diagnosis is essential for correct diagnosis of the disease, due to the strong similarity of ASF clinical signs and macroscopic lesions with those of other haemorrhagic diseases of pigs. Several effective tests are available to detect infectious virus, viral antigens, viral DNA or specific antibodies induced by the 22 p72 different ASF genotypes (World Organization for Animal Health, 2010b). The parallel detection of antigen and antibodies is crucial for establishing correct diagnosis and evaluating the progress of the disease control programme (Sánchez-Vizcaíno, 2006). This parallel diagnosis is not being currently developed in the affected area of Russian Federation and Trans Caucasian Countries, but should be soon implemented to delimit the ASF affected areas, control the progress of the disease, evaluate the successful of control programmes and definitively confirm the absence of carrier and recovered animals. African swine fever eradication without vaccination is possible, but difficult. Indeed, the eradication of ASF from Portugal and Spain proved that vaccination is not required for eradication even from countries where the disease has long been endemic (Arias and Sánchez-Vizcaíno, 2002b). However, success depends on implementing a good eradication programme approved by all the role players (farmers, veterinarians and policy makers), adapted to each specific scenario and adequately financially supported. For example, ASF eradication programme (1985–1995) was internationally funded with $72 million from the European Community over five years (Bech-Nielsen et al., 1995). Such a programme should provide accurate and timely information to farmers about the main sources of infection; monitor the movement of pigs and pork products; completely prohibit swill feeding practices; detect and eliminate positive animals, reservoir animals and carriers; increase biosecurity measures on farms; and provide reasonable economic compensation to farmers (Arias and Sánchez-Vizcaíno, 2002b). In the case of Sub-Saharan countries, although control and eradication programmes are cost and difficult, nowadays, there are unique solutions available against ASF, due to the absence of an effective vaccine. In such countries, where funds and resources are limited and insufficient for disease eradication, the perspective of the programme should not be the prompt eradication of the disease, but the improvement in pig husbandry systems and biosecurity measures, to achieve disease control and establishment of free disease areas in certain territories.

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Conflict of Interest Statement

All authors declare no conflict of interests.

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Review

African swine fever (ASF): Five years around Europe

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ABSTRACT

Since African swine fever (ASF) was re-introduced into Eastern Europe in April 2007, the disease has spread through five countries, drastically changing the European ASF situation. This re-introduction has significant implications for the affected countries, and it puts the European Union (EU) at serious risk of ASF introduction. Numerous factors are complicating the control of ASF in the Russian Federation and neighboring areas, particularly the absence of a coordinated control program, the abundance of backyard pig units with low or no biosecurity and the traditional use of swill feeding. All these risk factors are driven in turn by socio-economic, political and cultural factors. Moreover, the lack of clear information regarding the current situation of ASF in the Trans-Caucasus countries such as Armenia and Georgia may be increasing the risk of ASF spread into neighboring areas.

The ASF situation in Eastern Europe poses a constant risk of ASF entry into the EU, especially via routes that are difficult to control, such as wild boar movements, illegal movement of animals and animal products and movements of contaminated vehicles or other fomites. This paper reviews and discusses current ASF epidemiology in Eastern Europe, the factors that may contribute to disease endemicity in the area, the current challenges for disease control, and the risk of introduction into the EU.

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1. Introduction

African swine fever (ASF), one of the seven swine diseases notifiable to the World Organization of Animal Health (OIE), is causing significant problems on the European continent. The ASF virus (ASFV) is large, its genome and antigens are highly variable, and it is highly resistant when present in organic material and animal products. Several epidemiological cycles exist involving different hosts (wild and/or domestic pigs and/or soft ticks), and no effective vaccine is available. These are only a few of the main challenges for the effective control and

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eradication of ASF that help explain why it is considered one of the most complex infectious diseases of pigs (Sánchez-Vizcaíno et al., 2012).

For the last several decades, ASF has affected pig populations in southwestern Europe and particularly on the Iberian Peninsula, in addition to causing sporadic outbreaks in other European countries, such as Andorra (1975), Belgium (1985), France (1964, 1967 and 1974), Malta (1978), The Netherlands (1986) and Italy (1967, 1969 and 1993). ASF has also caused outbreaks on the island of Sardinia, where it has remained endemic since 1978 (EFSA, 2010a). In 1995, the disease was eradicated from the Iberian Peninsula, and until 2007 it was recorded only in Sardinia and nowhere else on the European continent. However, during the same period from 1995 to 2007, the disease spread extensively in many African countries, particularly in West Africa and on some islands previously free of the disease, such as Madagascar and Mauritius. Perhaps as a consequence of this ASFV increase in Africa, in conjunction with globalization, the economic crisis, and the use of contaminated garbage/swill to feed pigs, ASFV was re-introduced onto the European continent in 2007. This time, ASFV was introduced via Eastern Europe, specifically Georgia, from which it spread rapidly to the Russian Federation, Armenia, Azerbaijan and most recently to Ukraine (OIE, 2012a). This spread has significant implications not only for affected countries but also for neighboring ones, including the European Union (EU).

2. Evolution of ASF epidemiology in Eastern Europe

ASF was first recorded in Eastern Europe in 1977 in the Soviet Union, now the Russian Federation (GAIN, 2010). In the 2007 re-introduction, the disease was first detected in April near the port of Poti on the black sea; this outbreak was probably the sole source of ASF re-introduction and of all subsequent ASF cases in the area (Malogolovkin et al., 2012). The disease may have been introduced through transport of infected meat products on international ships, which were later fed to pigs (Beltran-Alcrudo et al., 2008), similar to what happened in the 1970s and 1980s in several Latin American and European countries, including Portugal, Brazil, and Cuba (Costard et al., 2009).

Within just two months, ASFV rapidly infected the naïve pig population of Georgia, causing the death of 68,000 animals or 19% of the susceptible population in the country (OIE, 2008). However, after August 2007, Georgia reported no more cases to the OIE. The second affected country in the area was Armenia, which officially notified the OIE in August 2007 but then stopped ASF notifications by the end of that year, mirroring the behavior of Georgia. Armenia later reported 4 ASF outbreaks in 2010 and 11 in 2011. These recent outbreaks affected wild boar populations within 30 km of the Georgian border, as well as domestic pig populations within 3 km of the border, increasing concerns about the ASF situation in Georgia.

Azerbaijan, another country in the Caucasus, was affected by a single outbreak of ASF declared in January 2008. The neighboring region of northwest Iran also reported the presence of infected wild boars in the scientific literature (Rahimi et al., 2010). The small numbers of pigs in these Islamic countries limited the ability of ASF to spread from Eastern Europe to countries farther east. The Russian Federation declared its first recent ASF outbreak in November 2007, along the border with Georgia, potentially as a result of contact with infected wild boar in that country (OIE, 2007). The presence of ASF in the Russian Federation poses the greatest danger to the EU and the rest of Europe, given the country's large pig industry, its large geographic size, and its proximity and extensive trade contacts with the EU. Based on official OIE reports (OIE, 2012b), the disease persisted and spread locally through wild boar populations around the Caucasus mountains during the first seven months of the outbreak. Unfortunately, in June 2008, the domestic pig population became infected and the disease began to spread throughout the country. During 2009 and 2010, the disease remained limited to southern districts, though it did reach some neighboring regions to the north. During 2011, ASFV moved farther north, causing more than 20 notified outbreaks far from the original endemic zone. During 2012 the disease continued to spread, giving rise to sporadic outbreaks only 87 km from the EU border. Some of these outbreaks affected wild boar and domestic pig populations in northern regions, producing new endemic areas, such as the Tver region close to Moscow, where a total of 34 ASF outbreaks were notified to the OIE between May 2011 and August 2012. These outbreaks are of serious concern to Russian authorities. Additional outbreaks in Eastern Europe have increased concerns in countries neighboring the Russian Federation, as well as in countries farther east. Nevertheless, no ASF-affected areas have been reported in the east of the Russian Federation as of November 2012 based on WAHID data (OIE, 2012d) (Fig. 1).

In July 2012 Ukraine officially notified the OIE of its first ASF outbreak, in which 3 of 5 pigs on a backyard farm died in the southeast of the country (OIE, 2012a). Following international norms, the Ukrainian government responded quickly to the outbreak, destroying the affected animals and implementing quarantine areas and control measures such as farmer compensation. This first detection of ASF in Ukraine, as well as the recent spread of the disease in northern and eastern regions of the Russian Federation, has increased concerns at the FAO that it may spread into neighboring areas such as Moldova, Kazakhstan or Latvia, where the abundance of pigs on family-type farms increases the risk of ASF introduction (FAO, 2012).

Eastern European countries have notified as many as 385 ASF outbreaks to the OIE, all caused by a single introduction of the virus in 2007 (Malogolovkin et al., 2012). During these outbreaks, more than 81,000 pigs (99.7% domestic animals, 0.3% wild boars) were infected and more than 297,000 animals were destroyed (OIE, 2012b). The disease has not affected all countries uniformly. Although Georgia has reported the highest number of infected pigs (70,522 infected in 60 outbreaks), the Russian Federation has reported the highest number of outbreaks (290) and the highest number of animals destroyed (274,729), corresponding to the largest economic losses. In fact, the total economic losses caused by

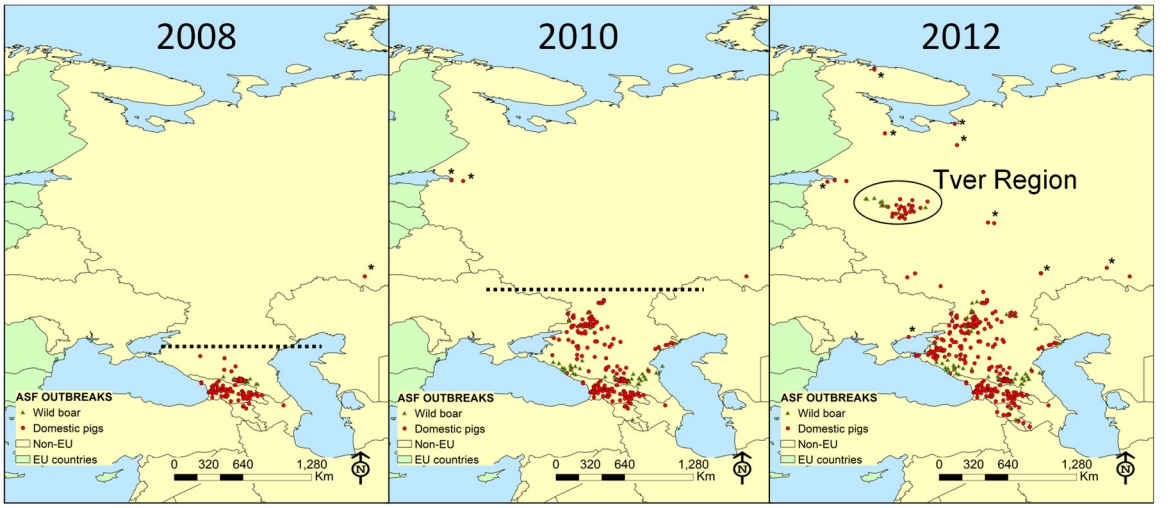

Fig. 1. Evolution of ASF epidemiology in Eastern Europe from 2007 to 19 July 2012. Dashed lines indicate the maximum northern latitude reached by ASF outbreaks in 2008, 2010 and 2012. Sporadic outbreaks occurring far from the southern endemic area are marked with asterisks (*). Own elaboration with data from WAHID database (OIE, 2012b).

ASF in the Russian Federation since its introduction in 2008 through 2011 have been estimated at approximately 7.6 billion roubles (240 billion USD) (Callaway, 2012).

The ASF epidemic in Trans-Caucasus countries has led to a huge decrease in the pig population (Fig. 2B). For example, from 2006 to 2010, the total number of pigs in Georgia fell by 70% and in Armenia by 56%. Similarly Azerbaijan lost 76% of its pig population, which, given the initially small population of 22,000, meant the near disappearance of pigs from the country (FAOSTAT, 2012). Azerbaijan reported only one ASF outbreak, which by itself led to the destruction of 4700 animals, or 25% of the pig population. Additional unknown factors may also have contributed to the drastic reduction in the pig population.

Conversely, during this same period of 2006–2010, the Russian Federation was unique in that its pig population increased by 3.7 million animals, despite the presence of the disease (Fig. 2A). However, this upward trend reversed after 2010, as both the pig population and the number of pig farms has been decreasing, especially in areas strongly affected by the disease. Paradoxically, these reductions may reflect efforts by Russian authorities to improve biosecurity in the pig industry and thereby fight against ASF. For example, the government now requires pig farmers to implant microchips in their animals. Since most small farms cannot afford these microchips, their numbers are expected to decline in favor of larger operations (Vorotnikov, 2012).

Affected countries have differed significantly in how rapidly they detect and notify the disease. In order to allow cross-country comparisons, data from official OIE reports were entered into a database (OIE, 2012d), including the date of the start of the outbreak and the notification date. The difference between these dates allows estimation of notification delays. For the period from 2007 to 2012, the Russian Federation has shown the fastest action, with the shortest mean number of days between the start of the outbreak and official notification of the disease to the OIE (mean 16.7, SD 20.8). Analysis of notification time in recent years reveals important changes in country behavior. For example, Armenia was among the countries with the fastest notification until 2011. However, during that year, notification time lengthened, reaching up to 242 days, revealing a more relaxed attitude in the notification system. Notification time in the Russian Federation

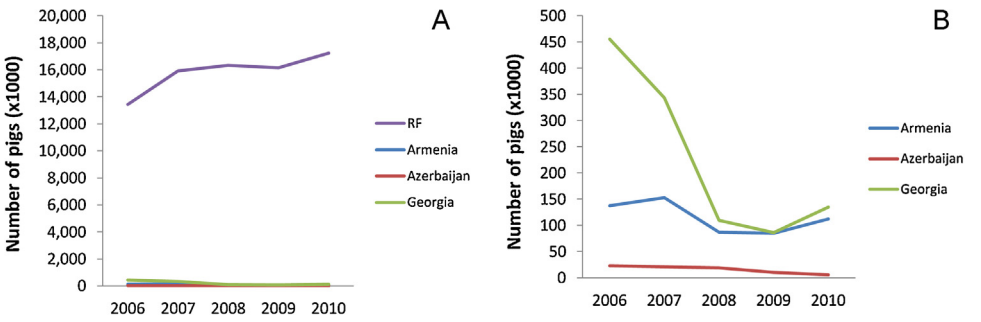

Fig. 2. Evolution of the pig population in all ASF-affected countries in Europe (A) or only in ASF-affected countries in the Trans-Caucasus region, excluding the Russian Federation (RF) (B). Own elaboration with data from FAOSTAT (FAOSTAT, 2012).

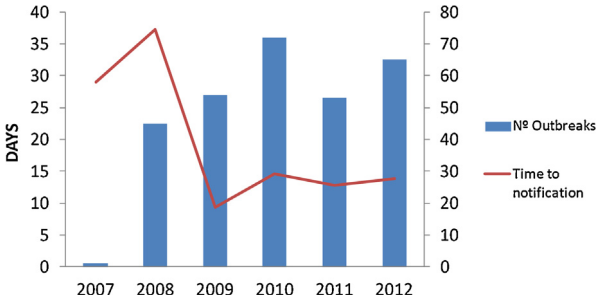

Fig. 3. Mean notification time and number of outbreaks in the Russian Federation since ASF re-introduction in Europe in 2007. Own elaboration with data from WAHID database (OIE, 2012d).

significantly improved (Kruskal–Wallis test, $p < 0.001$) between 2008 (median 14 days) and 2009 (median 5 days). This improvement most likely reflects implementation of surveillance and control programs in the country. Subsequently, however, median notification time increased from 5 days in 2009 to 10 days in 2012, which could reflect an increasing delay or lack of interest in disease notification (Fig. 3).

3. Challenges for ASF control in Eastern Europe

ASF continues to spread uncontrollably in the Russian Federation; it was recently introduced into Ukraine; and its presence is confirmed in Armenia and likely in Georgia. These developments show that efforts to contain ASF in Eastern Europe during the past five years have been far from successful. The disease has evolved to give rise to an even more intricate situation, reflecting a complex interaction of sanitary, economic, environmental and sociological factors in the region.

One of the main challenges for ASF control in the Russian Federation is the absence of a centralized program to combat the disease. This is particularly critical for such a large country with a large pig population, where ASF has already affected 26 regions, most of which share a border. This control program should provide updated information and education about the risk involving all stakeholders in the pig sector; it should link not only regional and central authorities, but also pig producers and public and private veterinarians in a joint fight against ASF.

Another significant challenge to ASF control, and probably the one most difficult to solve, is the illegal movement of animals and infected meat. Recent studies have revealed the influence of anthropogenic features such as road density on ASF outbreaks in the Russian Federation, suggesting that illegal trade of ASF-contaminated animals and products exists in the country (Gulenkin et al., 2011). Such trade may have caused the numerous outbreaks across the Federation during 2011 and 2012, as well as the regional spread of the disease across the southern federal districts (FAO, 2010) and the recent introduction into Ukraine (Rosselkhoznadzor, 2012). This illegal movement of meat is tightly linked with an important cultural factor that forms part of traditional pig breeding. In many areas of Eastern Europe, raising pigs in the backyard or on family farms with little or no biosecurity is quite common. In this kind of pig production, swill and other sub-products are commonly used to feed pigs because these materials are much more affordable than industrial feeds. This aspect of traditional pig breeding, reflecting the socio-cultural and economic characteristics of the area, is one of the most important factors in the spread and persistence of ASF in the Russian Federation. For that reason, farmers and breeders in the area should receive more information and education about the risk of swill feeding in order to reduce the negative consequences associated with it.

Another general socio-cultural problem relates to an apparent lack of interest in eradicating ASF, which may derive from several causes. Since ASF is not a zoonotic disease, it appears much less threatening to society than other animal diseases such as avian influenza or bovine spongiform encephalitis. The Russian Federation and Trans-Caucasus countries are not pig-exporting countries, so the international trade restrictions imposed on ASF-affected countries do not pose economic problems for them. At the same time, the mortality caused by ASF in Eastern Europe has probably fallen over the last five years, and the disease does not spread as rapidly as foot-and-mouth disease, classical swine fever or influenza. In addition, backyard farmers probably often choose not to report cases in order to avoid the loss of their pigs (Callaway, 2012). All these factors likely reduce social and governmental concern about ASF, and they may lead to non-compliance with established control measures. The lack of economic compensation to farmers for lost animals probably further contributes to non-compliance. All these factors, together with ASF infection of wild life and the potential presence of tick hosts are seriously complicating ASF eradication efforts, contributing to the endemicity of the disease in the area.

4. Risk of ASF entry into the EU and probable consequences

The uncontrolled evolution of the disease in Eastern Europe, particularly in 2011 and 2012, and the geographical and commercial proximity of the Russian Federation and Ukraine to the EU have increased the risk of ASF introduction into the Union. Nevertheless, the risk differs substantially among EU countries, particularly when the different routes for potential ASF introduction are taken into account (Mur et al., 2012a). Although export of pigs and pig-derived products from ASF-affected countries is illegal, ASFV can leave these countries and enter the EU through other routes that are less easy to control.

One of these risky routes is wild life. Wild boar populations have been affected by ASF outbreaks in the endemic southern areas of the Russian Federation and Trans-Caucasus region since the virus was re-introduced into Europe (OIE, 2007). More recently, in 2011 and particularly in 2012, infected wild boar were found for the first time far outside the southern endemic region, in the Tver region near Moscow (Fig. 1). There the disease seems to have established itself in domestic and wild boar populations (OIE, 2012c). This new situation implies higher risk for EU countries bordering the Russian Federation, such as Finland, Estonia, and Latvia. The

evolution of ASF in the Tver should be monitored in order to update surveillance and contingency plans in the area.

Another important route difficult to monitor and control is the illegal trade of animals and products from the Russian Federation into other countries. Such trade is driven by differences in the price of pig meat, as well as the lack of appropriate control measures on the roads. In addition, transport fomites pose a risk to the EU. Livestock vehicles arriving on farms in the Russian Federation can come into contact with ASFV and bring it back to the EU; international planes and ships can carry infected food (Mur et al., 2012a). The long survival time of ASFV in meat products (1000 days in frozen meat), putrefied blood (15 weeks at room temperature) and feces (11 days at room temperature) (EFSA, 2010a) increases the risk of transmission to ASF-free areas.

The probability that ASF may enter the EU, and the likely consequences of introduction, vary significantly from region to region, depending on such characteristics as the existence and density of wild boar populations in the area. Thus it is very important to know the density (by surface area) of wild boar populations and their contacts with potential sources of the disease, such as backyard pigs and other free-range pigs, since wild boar by themselves are unable to maintain the disease for long periods (Mur et al., 2012b).

Risk of ASF endemicity and spread will depend on the number, density, and characteristics of pig farms with low biosecurity. The consequences of an ASF outbreak affecting one area with a single industrial farm of 3500 pigs will be completely different from those of an outbreak affecting a rural area with 50 family farms and 70 backyard pigs. The number of affected pigs may be the same, but the risk of endemicity and further spread is completely different. Control measures are much easier to implement on industrial farms than on smaller farms, though they cost more on industrial farms. Lack of understanding among small farmers of the risk of disease transmission makes it difficult to ensure timely detection of the disease, complicating the control of outbreaks. In addition, backyard and family farms should be checked individually, increasing the time needed for monitoring and consequently the risk of ASF spread.

Another important factor for the establishment and spread of ASF, though not for its entry, is the presence of competent ticks that replicate and maintain the virus for long periods (Boinas et al., 2011). In Sardinia, no soft ticks have been found so far, but this negative result should be confirmed. *Ornithodoros* species have been found in Georgia and Armenia (EFSA, 2010b), but their presence and potential role in ASF transmission is not fully understood.

Perhaps the most important point for ASF control is the need for an adequate early detection system and contingency plans to ensure rapid response in different scenarios. This system should be prepared and approved in advance, and must comprise education and training, as well as appropriate surveillance and contingency planning. Training and education should take the form of information campaigns that inform all stakeholders about the current risk of introduction and the main characteristics of the disease. Like the surveillance system, training and education efforts should be active and based on current risk assessments. The contingency plan should be complete and detailed enough that everyone will know when and how to act during an outbreak.

Finally, while the presence of ASFV in the Russian Federation and neighboring Trans-Caucasus countries poses a particular risk to the EU and the rest of Europe, the presence of ASFV in Africa also poses a risk to Europe as well as to potentially any other country, including in Asia. Indeed, the high prevalence of ASFV on the African continent in recent decades, establishing in previously disease-free territories (Sánchez-Vizcaíno et al., 2012), means that all countries should be aware of the risk of spread and should update their contingency plans. The damage in Eastern Europe caused by a single ASFV introduction into Georgia should serve as a reminder of the importance of adequate planning and control measures.

5. Conclusion

The ongoing and rapid spread of African swine fever in Eastern European countries has changed the European epidemiological situation and poses a risk for the EU. Although some measures have been implemented in Eastern Europe, the disease is not under control in the Russian Federation or Armenia and probably not in Georgia. ASF persists in the area because of a wide range of economic and socio-cultural factors that should be taken into account in the design of effective eradication programs. In addition to the risk of ASF spread from Eastern Europe, the risk of ASF spread from the African continent should also be taken into account, given the high disease prevalence there.

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OBJETIVO 2: Caracterización del riesgo de introducción del vPPA en la UE para dos de las vías de entrada más importantes y desarrollo de una herramienta modular para la integración de análisis de riesgo.

La reciente entrada y difusión incontrolada de la PPA en Rusia y la región del Cáucaso alcanzando las fronteras de la UE, ha aumentado la preocupación respecto a la posible de introducción del vPPA en la UE. Las principales vías por las que esto podría ocurrir son: comercio legal de animales vivos y productos, entrada ilegal de productos de cerdo, rutas asociadas al transporte y movimiento de jabalíes.

En esta tesis se desarrollaron análisis para estimar el riesgo asociado a dos de estas vías (comercio legal de cerdos vivos y riesgo asociado al transporte) (objetivo 2.1), y se elaboró una herramienta global para la visualización y análisis conjunto de los resultados de todas las vías anteriormente mencionadas (objetivo 2.2).

Objetivo 2.1

El comercio legal de cerdos ha sido históricamente, una de las vías más importante para la entrada de enfermedades exóticas animales en la UE. Para estimar la probabilidad mensual de la entrada del vPPA en cada uno de los países de la UE por esta vía, se empleó un modelo estocástico cuantitativo. Los resultados de este modelo sugieren una probabilidad de entrada del vPPA por esta vía para el global de la UE, de $5.22*10^{-3}$, que corresponde aproximadamente con un brote cada 192 años. El riesgo de entrada por cerdos vivos fue mayor en Polonia, especialmente durante los meses de noviembre y diciembre.

Bajo el concepto de riesgos asociados al transporte (RAT) se incluyeron tres tipos de rutas: camiones de regreso, residuos de barcos internacionales o residuos de aviones internacionales. Debido a la ausencia de información disponible para estas vías, se empleó un modelo semi-cuantitativo de riesgo, basado en la combinación ponderada de los factores de riesgo. Los pesos relativos para la combinación de los distintos factores de riesgo fueron obtenidos por encuesta de opinión de expertos. Los resultados del modelo indican que el riesgo relativo de entrada del vPPA a través de RAT es bajo en la mayoría de países de la UE (16), aunque algunos países como Polonia y Lituania concentran valores de riesgo elevados. La ruta de camiones de regreso es la que suponen un mayor riesgo de las RAT analizadas.

Objetivo 2.2

Estos dos análisis, sumados a los de otras tres vías analizadas en el marco de un proyecto europeo, en las que también colaboré en su desarrollo, fueron integrados de manera conjunta en una herramienta modular aquí desarrollada. Esta herramienta permite la visualización y análisis global del riesgo relativo de introducción del vPPA en la UE. En base a esto, el 48% de países de la UE presentaron un riesgo relativo elevado para al menos una de las vías analizadas. Destacan cuatro países, los cuales presentaron el valor de riesgo más elevado para alguna de las vías analizadas: Bulgaria para el comercio legal de productos durante el periodo de alto riesgo (HRP); Finlandia para la vía de jabalíes y Eslovenia y Suecia para el comercio de cerdos durante el HRP. La distribución del riesgo varió considerablemente entre las vías, existiendo vías en las cuales el riesgo se concentró en unos pocos países (RAT), mientras que otras vías presentaron 4 o 5 países con riesgo elevado (comercio legal de cerdos, importaciones ilegales y jabalíes).

Los análisis de riesgo desarrollados en esta tesis proporcionaron unos resultados de gran interés para la actualización y mejora de los planes de vigilancia y control de PPA en la UE, mostrando las vías y países en las cuales el riesgo de entrada es más elevado. La adaptación de los planes a estos resultados repercutiría en una mejora de los programas de detección temprana basados en riesgo permitiendo una mejor prevención y control de las potenciales incursiones del vPPA en la UE.

Artículos científicos:

- Incluidos en la tesis:
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    - Mur L., Martinez-Lopez B., and Sanchez-Vizcaino JM., 2012. **Risk of African swine fever introduction into the European Union through transport-associated routes: returning trucks and waste from international ships and planes**. *BMC vet res*, 8, 149.
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- No incluidos en la tesis:
    - Costard S., Jones BA., Martinez-Lopez B., Mur L., de la Torre A., Martinez M., Sanchez-Vizcaino F., Sanchez-Vizcaino JM., Pfeiffer DU., Wieland B., 2013. **Introduction of African Swine Fever into the European Union through Illegal Importation of Pork and Pork Products.** *PLoS ONE* 8(4): e61104.
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ORIGINAL ARTICLE

Quantitative Risk Assessment for the Introduction of African Swine Fever Virus into the European Union by Legal Import of Live Pigs

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Summary

The recent incursion and spread of African swine fever virus (ASFV) in the Russian Federation and Caucasus region, close to European Union (EU) borders, have increased the concerns regarding the probability of ASFV introduction into the EU. There are many potential routes of ASFV entry into EU, but here we specifically aimed to assess the probability of ASFV introduction by legal trade of pigs, which historically has been one of the most important ways of exotic diseases introduction into the EU. A stochastic model was used to estimate the monthly probability of ASFV introduction for each country of the EU. Results of this model suggest an annual probability for ASFV introduction in the whole EU by this way of $5.22*10^{-3}$, which approximately corresponds with one outbreak in 192 years. The risk of ASFV introduction via live pigs was highest in Poland (69%), particularly during the months of November and December. As expected, Russian Federation is the country that most contributes to this risk, representing 68% of the overall annual risk. Methods and results presented here may be useful for informing risk-based surveillance and control programmes and, ultimately, for prevention and control of potential ASFV incursions into the EU.

Introduction

African swine fever (ASF) is a notifiable, viral infectious disease which affects domestic pigs and wild suids and results in severe sanitary and economic consequences in affected countries (Penrith and Vosloo, 2009). ASF is a haemorrhagic disease caused by a DNA virus, ASF virus (ASFV), which is a unique member of the *Asfarviridae* family (Dixon et al., 2005). ASFV affects all ages and breeds of pigs and produces a wide range of clinical signs, varying from a peracute/acute (90–100% of mortality) to a chronic form with inapparent carriers. Transmission of ASFV may occur either by direct contact with infected animals or by indirect contact with contaminated meat or fomites or with biological vectors (i.e. ticks of genus *Ornithodoros*). Because there is no vaccine available for ASFV, the prevention of the disease entry into disease-free areas is critical and mainly based on the avoidance of the introduction of potential infected pigs or pig products, as well as appropriate disposal of pork waste from aircraft and ships and other fomites (Sánchez-Vizcaíno, 2006).

African swine fever virus was firstly described by Montgomery in Kenya, in 1921. Since then, the disease has become endemic in most sub-Saharan countries. In 1957, ASFV reached Europe, resulting in outbreaks in Portugal, Spain, France, Belgium, Malta, Netherlands and Italy, and it remained endemic on the Iberian Peninsula from 1960 to 1995. The sanitary and economic losses of this endemic period were enormous, with more than 2 323 018 slaughtered pigs (MAPA, 1966–1994) and, at least, €65

million losses in Spain (Arias and Sanchez-Vizcaino, 2002). Further outbreaks occurred in Europe and Central and South America during the 1970s and 1980s, but all of them were eradicated, except in Sardinia (Italy), where the disease still persists today. The epidemiological situation changed dramatically in 2007, when ASFV was reintroduced to the European continent, on this occasion in the Caucasus region. The hypothesized route of ASFV introduction was contaminated swill from ships that was ingested by free roaming pigs near the port of Poti (Georgia) (Beltran-Alcrudo et al., 2008). After this first incursion into Georgia, the disease has spread to Armenia, Azerbaijan and the Russian Federation (RF) with more than 260 notified outbreaks and 76 000 affected animals in three years (OIE, 2011a). The economic consequences of the ASF epidemic for the Russian pork industry, until October 2009, were estimated at RuR 25–30 billion ($US 0.8–1 billion)) (USDA, 2010). Furthermore, two of the outbreaks occurring in October 2009 and in December 2010, were located very close to the EU border (approximately 150 km) (OIE, 2009a, 2010a). The likelihood of ASF becoming endemic in RF has been estimated as high, because of the uncontrolled course of the disease, the demonstrated active spread of ASFV in the wild boar population, and the large volume of uncontrolled trade of pigs and pig products taking place across the country (EFSA, 2010; Wieland et al., 2011).

An important route for the introduction and spread of animal diseases is the movement of live animals or their products. For that reason, the World Trade Organization (WTO) and the World Organization for Animal Health (OIE) encourage the countries to follow the guidelines specified in the Sanitary and Phytosanitary Measures Agreement (SPS), as well as to perform risk assessments to support their preventive and control policies. Following these guidelines, the trade of pigs and pig products from an ASFV-infected country or zone to a disease-free country or area is not permitted (OIE, 2009b). Once a region is declared as ASFV infected, an immediate ban of movements of live pigs and pig products is implemented. Considering this principle, the possibility of ASFV introduction by legal trade of live pigs or pig products may only occur during the 'high-risk period' (HRP), which is the period of time from the initial infection (i.e. index case) until the official diagnosis and notification of the disease for a region (Horst et al., 1998). During the HRP, trade of pigs and pig products are not restricted and ASFV can freely spread to other areas/countries. Considering the distances covered and frequency of animal movements, a HRP of 1 or 2 weeks may result in an extensive spread of a disease. In fact, the movement of animals during the HRP has been responsible for the spread of diseases such as foot and mouth disease (Bouma et al., 2003); classical swine fever (Greisser-Wilke et al., 2000) or bovine spongiform encephalopathy (Smith and Bradley, 2003) within EU regions in the last 10 years.

The EU is the second largest pig producer in the world, behind China, and the second largest exporter of pigs behind United States (FAO, 2009) (e.g. in 2009 157 043 100 kg live pigs were exported) (EUROSTAT database). The introduction and spread of a disease such as ASFV into the EU would severely impact negatively on the EU pig sector and the economy of the EU. Given the high risk of endemicity of ASFV and continued spread in the Russian Federation (EFSA, 2010), there is a need to estimate the potential risk of ASFV introduction into the EU as well as to define the geographical areas and time periods at highest risk so as to be able to prevent potential ASFV incursions and support future policies.

This study aimed at quantifying the risk of ASFV introduction into the EU associated with legal movement of pigs. The framework proposed here was planned to be combined with risk assessments for other ASFV routes of introduction into the EU (i.e. import of pig products, illegal trade of pigs and pig products, fomites, etc.) and may be easily updated and adapted for other regions or countries.

Methods

Data sources and unit of analysis

The selected spatial and temporal unit of analysis was country and month, respectively. This level of aggregation was assumed to be the most appropriate to support EU policies and the most suitable, given the available information. Information regarding the number of live pigs imported by month and country during the last 5 years (2005–2009) was obtained in the EU official database Trade Control and Expert System (TRACES). Data from the Statistical Office of the European Union (EUROSTAT database) was used to validate the model in the sensitivity analysis. The number of destination countries (d) considered in the current analysis was 27, corresponding to the 27 member countries of the EU; and the number of origin countries (o) analysed was five, those for which imports of live pigs were registered for the last 5 years.

Model formulation

The probability of having at least one outbreak of ASF, namely the probability of introduction (PI), in one of the 27 EU countries because of import of an ASF-infected (but non-detected) pig during the HRP was estimated per country of origin of imported pigs ($o = 5$), per month ($m = 12$) and by country of destination ($d = 27$), assuming a binomial process (OIE, 2010b) of the form:

$$PI = \sum 1 - (1 - p_{odm})^{n_{odm}}$$

where n_{odm} is the number of live pigs imported from country of origin o to country of destination d during the month m, and p_{odm} is the probability of introducing an infected pig from country o to country d during month m and having an effective contact from an infected to a susceptible pig in the destination country d.

The probability p_{odm} was estimated as the product of three conditional probabilities. The probability of importing an ASFV-infected pig from country o before detection of infection (P_1); the probability that an ASFV-infected pig survives (P_2) and the probability that an imported pig has a contact with a susceptible animal in the country of destination d (P_3) which results in transmission of ASFV. This approach was similar to the model used for the estimation of introduction of classical swine fever in Spain (Martínez-López et al., 2009). All assumptions, input values, parameterization and references used in the model are presented in Table 1 and are explained in detail in the next sections.

We have to assume that model results are always conditioned to the quality and update of the data available. In this case, results will be conditioned to historic data trade records and the available information from the current situation of the disease. Other three major assumptions used in the model are explained in the following paragraphs for better comprehension of the model: regarding the probability of infection in the source country, if there were no reports of ASF in the considered country, 88 years without disease were assumed (since the initial report of the virus, in 1921). For the estimation of ASF undetected outbreaks, historical data from previous ASF outbreaks was used, assuming a similar surveillance capacity in all the origin countries. Related with the disease, for all the measures and probabilities (survival, detection), it was assumed that ASF will be present in an acute form of the disease, as it is currently occurring in Caucasus region and Russian Federation and as it occurred in the first stages of infection in European countries. The model was constructed using @Risk version 5.5 (Palisade Corporation, Newfield, NY, USA) in Microsoft Excel (Microsoft Office 2007 Professional Edition). Results were obtained after 10 000 iterations using the Monte Carlo simulation approach, and maps to visualize the results were generated using ArcGIS 9.2 (ESRI).

Probability of selecting an ASFV-infected pig from country o before detection of ASFV infection (P_1)

The probability of selecting an ASFV-infected pig from origin country o to be exported to EU country d before detection of ASFV infection during a particular month m (P_1) was modelled using a beta distribution. The shape of this distribution was given by the number of estimated infected pigs prior to ASF detection in the country of origin o, and the total number of pigs in country o (OIE, 2011b).

The number of infected pigs in country o was estimated using the data available on the epidemiological history of the disease and as a result of multiplying four independent parameters (Table 1). First, we calculate the probability of having a not yet detected ASFV epidemic in the source country (Po), which is the probability of country o being infected (but not detected) in month m. The probability Po was parameterized using a beta distribution of the form Beta (α_1, α_2), where α_1 is the number of months with at least one undetected ASF outbreak in the country of origin, and α_2 is the number of months considered in the analysis (i.e. number of months for which information was available in the OIE database (OIE, 2011a,c).

Once the probability of infection in country o in month m was defined, the most likely number of undetected outbreaks in country o in month m was estimated (Ou). The parameter Ou was estimated by considering the number of undetected outbreaks prior to the first notification of the disease to the OIE (OIE, 2011a) and was an estimation of the most likely number of infected but not detected pig herds before official notification of the disease. The value of Ou was parameterized by using a pert distribution of the form Pert (minimum, most likely, maximum).

The number of pigs and the number of pig herds in source country o was used to estimate the average herd size in country o (To). The number of pigs and pig herds was parameterized by using a normal distribution of form Normal (μ, σ), where μ is the average number of pigs (or pig herds) and σ is the standard deviation of the number of pigs (or pig herds) in country o during the last 5 years, respectively (OIE, 2011b; FAOSTAT, 2011; Statistics of Russian Federation, 2011).

Finally, we estimated the intraherd ASFV prevalence (Hp), which represents the proportion of ASFV-infected animals in an infected (but not detected) herd. This parameter was modelled by a pert distribution of the form Pert (min, most likely, max) with min, most likely and max values of 0.022, 0.4 and 0.8, respectively, which were obtained from the published literature (Wooldridge et al., 2006) and field observations from current outbreaks in Russian Federation (Blagodarsnosti, 2011).

Probability of survival of selected ASFV-infected pig (P_2)

The probability P_2 is the product of two independent probabilities, which are the probability that an infected pig survives ASFV-infection (Ps), and the probability that a pig survives the transport from the country of origin to the country of destination (Pt). The probability Ps, was calculated by using the mortality rates of different virus isolates

Table 1. Description and parameterization of model inputs

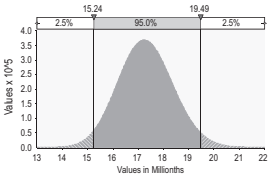
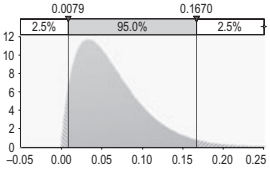
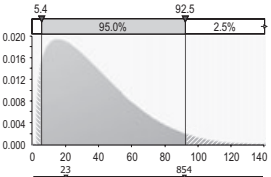
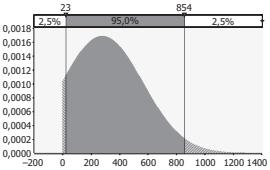
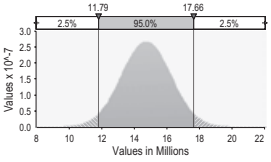
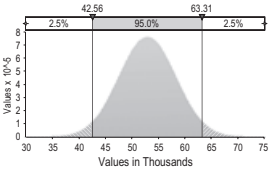
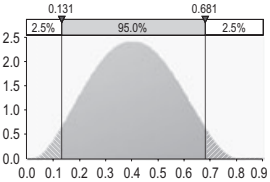
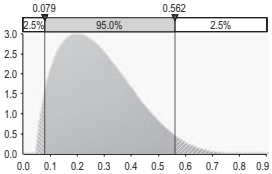
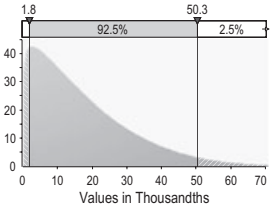
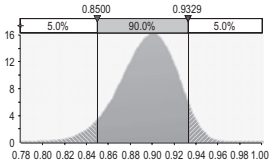
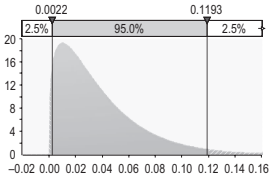
Notation	Definition	Source	Parametrization	Data values for Russian Federation (September)
P_1	Probability of selecting an ASF-infected pig from country *o* in month *m* before detection of ASFV infection	NI = *Po* × *Ou* × *To* × *Hp*No = pig population	Beta (α_1, α_2)α_1 = NI + 1α_2 = No−(NI + 1)	
Po	Probability of infection in country of origin	OIE (2011a), OIE (2011c)Where: *X*: number of outbreaks by month; *M*: number of months considered	Beta (α_1, α_2)α_1 = *X* + 1α_2 = *M*−(*X* + 1)	Beta (2, 30) 
Ou	Number of undetected outbreaks before official notification	OIE (2011a)	Pert (min, most likely, max)	Pert (3.2,15.6,153.2) 
To	Average herd size in country *o*		Normal = No/So	
No	Pig population size in country *o*	OIE (2011b), FAOSTAT (2011)	Normal (μ, σ)	Normal (14728567.5, 1497724.4) 
So	Number of pigs establishments in country *o*	OIE (2011b), Statistics of Russian Federation (2011)	Normal(μ, σ)	Normal (52934.5, 5293.5) 
Hp	Intraherd prevalence	Wooldridge et al. (2006), Blagodarsnosti (2011)	Pert (min, most likely, max)	Pert(0.022,0.4,0.8) 
P_2	Probability of survival of selected ASFV-infected pig	*Ps*Pt*		

Table 1. *Continued*

Notation	Definition	Source	Parametrization	Data values for Russian Federation (September)
Ps	Probability that an ASF-infected pig survives infection	Spickler and Roth (2006)	Pert (min, most likely, max)	Pert (0.05,0.2,0.8) 
Pt	Probability of ASFV-infected pig surviving transport	Murray and Johnson (1998)	Pert (min, most likely, max)	Pert (0.0005, 0.0027, 0.092) 
P_3	Probability of an imported ASFV-infected pig comes into contact with other domestic pigs in the destination country resulting in disease transmission	$P_q + [(1-P_q)*P_u]$		
P_q	Probability that pigs were not quarantined	Martínez-López et al. (2009); L. Romero, personal communication	Beta (α_1, α_2)	Beta (130.7, 15.4) 
P_u	Probability that an ASF-infected animal remains undetected during quarantine	Martínez-López et al. (2009); L. Romero, personal communication	Beta (α_1, α_2)	Beta (1.3, 34.2) 
n_{odm}	Imports of live pigs from country *o* to EU country *d* during month *m* (during last 10 years)	TRACES database	Normal (μ, σ)	
p_{odm}	Probability that an ASF-infected pig from country *o* enters a farm in country *d* during month *m*	$n = n_{odm}p = P_1 \times P_2 \times P_3$	Binomial (n, p)	

described in the literature (Spickler and Roth, 2006). The probability of surviving the transport (*Pt*) was also obtained from the literature (Murray and Johnson, 1998). Both probabilities were modelled by using pert distributions.

Probability that an imported ASFV-infected pig comes into contact with other domestic pigs in the destination country d (P_3) resulting in disease transmission

Whereas P_1 and P_2 were used to estimate the probability of release of an ASFV-infected pig, P_3 measured the probability of exposure of a susceptible pig in the EU. Because we considered only live pigs imported into farms (not to slaughterhouses) in our analysis, the value of P_3 only depended on biosecurity measures (i.e. quarantine) applied in the destination farms and was estimated as follows:

$$P_3 = P_q + (1 - P_q) * P_u$$

where P_q is the probability of not quarantining an imported pig and P_u is the probability of not detecting an

ASFV-infected pig when performing quarantine. Because of the absence of available data regarding the biosecurity practices for imported pigs applied in each of the EU countries, the probability of P_q for all EU countries was assumed to be the same as the one estimated for Spain and published in Martínez-López et al. in 2009. This was considered to be a reasonable standard value, based on the assumption that similar to Spain, other EU countries apply medium to high level of biosecurity measures for pigs imported from third countries.

The probability of not detecting an ASFV-infected pig by clinical signs during quarantine (P_u) was assumed to be the same as for classical swine fever virus (CSFV) (Martínez-López et al., 2009), as the diseases have similar clinical signs (Sánchez-Vizcaíno, 2006).

Sensitivity analysis

The effect that variability and uncertainty on input data and model parameters has on model outcomes was evaluated by using sensitivity analysis. Most quantitative risk assessment models developed for the European Union use official EU statistics (i.e. EUROSTAT) as the source of information for animal movement records. To compare the outcomes of the model using the more accurate TRACES data and the EUROSTAT information, the model was run using EUROSTAT data from the last 5 years. No other input parameters were changed.

In parallel, because of the large number of input parameters used in the model, a two-step sensitivity analysis was performed. First, regression coefficients (β_i) between each input and the annual probability of ASFV introduction in the EU were calculated to quantify the influence that changes in input values have on model outcomes (Vose, 2000). Second, the inputs that were most likely to influence the final results ($\beta_i \geq 0.1$) were further analysed using a one-way sensitivity analysis, in which their base values were changed in eight consecutive steps, from a minimum of 50% of reduction to a maximum of 50% increase. Results are reported by graphs and spider plots (Fig. 1).

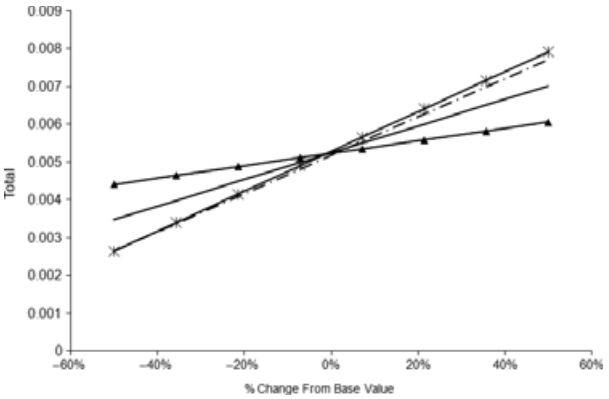


Fig. 1. Sensitivity analysis. Graph represents changes in the overall probability of African swine fever virus introduction into the EU by legal imports of live pigs as a result of variation in the most influential parameters of the model ($\beta \geq 0.1$): Probability of survival to ASF infection (⁎); intraherd prevalence (— · —); undetected outbreaks in Russian Federation (RF) (—); probability of infection in RF in November and Imports from RF to Poland (▲).

Results

Probability of having at least one ASF outbreak in the EU because of import of live pigs

The overall mean (95% PI) annual probability of ASFV introduction into the EU by legal import of live pigs was $5.22*10^{-3}$ ($6.06*10^{-4}$, $1.84*10^{-2}$), which approximately corresponds, if the underlying epidemiological conditions are constant, to one outbreak in 192 years, on average.

The probability of ASFV introduction into the EU was highest for Poland ($3.63*10^{-3}$), followed by the United Kingdom ($6.18*10^{-4}$) and Germany ($3.41*10^{-4}$) (Fig. 2). In fact, Poland represented 69% of the overall annual risk of ASFV introduction into the EU (27 analysed members).

Analysing the monthly distribution of the risk in these three high-risk countries, we observe that most of the risk for Poland and Germany is concentrated in one month. Specifically, the 46% of the annual risk in Poland is concentrated in November, whereas the 42% of the annual risk in Germany is concentrated in December. Conversely, the risk of introduction into United Kingdom is widely distributed in different months (February, October, December, April, August…), without any predominant risky month.

As expected, the country that contributed most to the risk of ASFV introduction into EU was the Russian Federation ($3.53*10^{-3}$), representing 68% of the overall annual risk (Fig. 3). Most part of this risk (47%) is concentrated during the month of November, coinciding with the highest risk of introduction into Poland.

The estimated probability for ASFV introduction into the EU was higher during the months of November ($1.75*10^{-3}$) and December ($7.29*10^{-4}$) (Fig. 4). These two months represented approximately 47% of the annual risk.

Sensitivity analysis

When using EUROSTAT instead of TRACES for input data, the ASFV risk of introduction into the EU through legal imports of live pigs was about two times higher ($1.14*10^{-2}$) than with TRACES ($5.22*10^{-3}$).

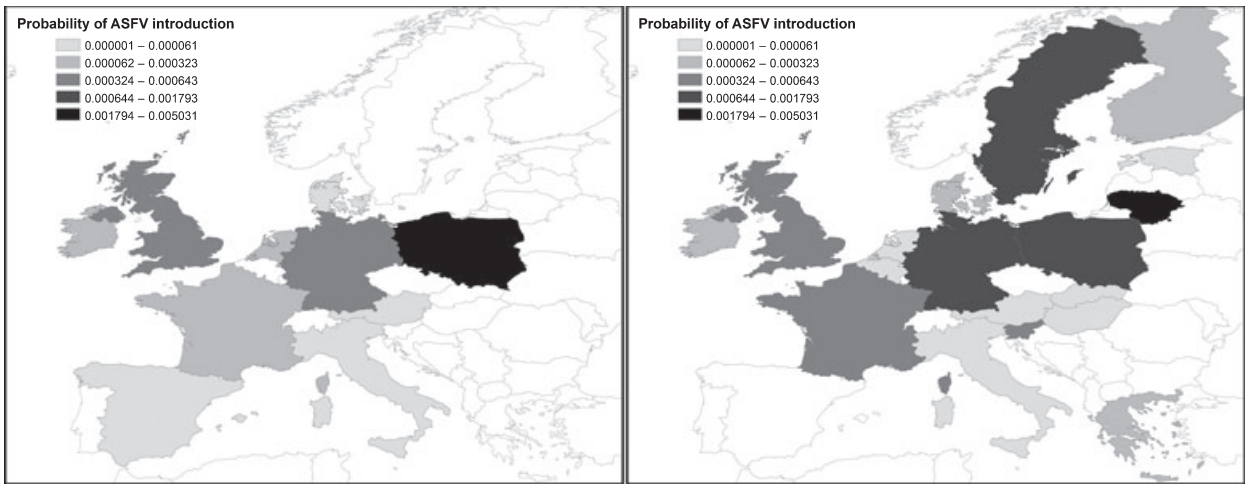


Fig. 2. Probability of African swine fever (ASFV) introduction into the EU by legal trade of live pigs by country of destination using different animal movement databases (TRACES left, EUROSTAT right).

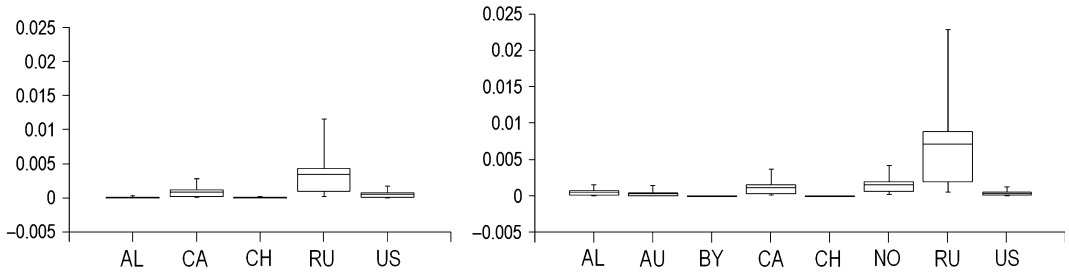


Fig. 3. Probability of African swine fever (ASFV) introduction into the EU by legal trade of live pigs by country of origin using different animal movement databases (TRACES on left side, EUROSTAT on right). AL, Albania; AU, Australia; BY, Belarus; CA, Canada; CH, Switzerland; NO, Norway; RU, Russian Federation; US, United States.

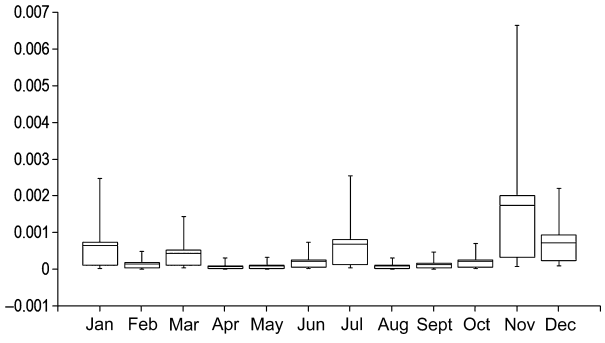


Fig. 4. Monthly probability of African swine fever (ASFV) introduction into the EU by legal trade of live pigs using the TRACES database.

Regarding the countries of origin, EUROSTAT registered eight countries importing pigs into the EU, whereas TRACES registered only five countries. In both cases, the Russian Federation was the country that posed the highest risk for the EU, representing 63% and 68% of the total annual risk based on EUROSTAT and TRACES databases, respectively.

Destination countries within the EU that concentrated the highest risk for ASFV introduction were Lithuania and Poland, based on EUROSTAT data and only Poland when using TRACES data. In both cases, United Kingdom and Germany were included as two of the five countries with highest risk for ASFV introduction (Fig. 2).

With respect to the seasonal variation in the risk of ASFV introduction into the EU, there were important differences when comparing both databases. Only November was considered to be higher risk with both data sets (Fig. 4).

The first step of the sensitivity analysis for input parameters allowed identifying seven parameters with the strongest influence on the model results ($\beta_i \geq 0.1$). However, the one-way sensitivity analysis revealed that model outcomes were only sensitive to changes in three of these parameters, which were the probability of survival of an

ASFV-infected pig, the intraherd ASFV prevalence and the number of outbreaks in the Russian Federation before the detection of the disease (Fig. 1).

Discussion

To the best of our knowledge, this is the first study quantifying the risk of ASFV introduction into the EU by legal import of live pigs. Moreover, this is the first study to specifically address the spatial and temporal variation of such a risk. The identification of geographical areas and time periods at highest risk of ASFV introduction can help update and improve the effectiveness of the surveillance programmes for the prevention and control of potential ASFV incursions into the EU.

Movement of live animals is certainly one of the main routes for disease introduction into free-areas (Ortiz-Pelaez et al., 2006; Zepeda et al., 2001). If we consider how fast and actively infected animals and animal products move in our global world, it is not difficult to imagine how fast an infected but non-detected animal can spread disease to remote regions or countries. In order to minimize risks related with animal trade, OIE and other international organizations are continuously stressing the need to develop risk assessments aimed at preventing and minimizing as much as possible the potential introduction of diseases into free areas.

The risk assessment presented in this study followed the internationally accepted guidelines recommended for the development of quantitative import risk assessments (OIE, 2010b), including the performance of a sensitivity analysis for model validation, and detailed description of the assumptions and parameterization of the inputs. This approach was adopted to enhance the transparency of the model development process in order for the model results to be useful for the decision-making process. A complete and accurate consequence assessment should be further developed in order to complete the risk-assessment process for estimation of the adverse direct and indirect socioeconomic consequences associated with a potential introduction of the disease in the EU.

The incursion of OIE-notifiable diseases into free areas, such as ASF into the EU, which is a large producer and trader of pigs and pig products, would certainly impact the economy of the region. For this reason, the number of studies intended to evaluate and quantify (when possible) the potential risks for disease introduction into free areas has increased in the last years. Recent examples found in literature are the quantitative risk assessment for classical swine fever virus (CSFV) introduction into the Danish pig population (Bronsvoort et al., 2008); the quantitative risk assessment for CSFV introduction by import of live pigs into Spain (Martínez-López et al., 2009); the model presented by de Vos et al. in 2004 to quantify the risk of CSFV introduction into Member States of the EU; and the quantitative assessment for highly pathogenic avian influenza introduction into Spain (Sanchez-Vizcaino et al., 2010), among others. The framework presented here not only quantifies the overall risk of ASFV introduction into the EU but also identifies geographical areas and time periods at increased risk of ASFV introduction, which could be useful to develop targeted risk-based surveillance and control strategies.

Accuracy and quality of the data used to parameterize the model determines the accuracy and quality of the outputs obtained. The stochastic model presented here uses the most recent and detailed information available (i.e TRACES system), but it needs to be acknowledged that it is a dynamic set of information which will change over time and sometimes access to such information is restricted to researchers. The model presented here was structured so that it can be easily updated/adapted to specific conditions/information available in the different EU countries. However, most of the quantitative models developed until now have used EUROSTAT statistics instead of TRACES, which usually is less detailed/disaggregated and less reliable than TRACES information, which is the official system for movement records in the EU. Results obtained from the sensitivity analysis indicate that, at least in this current model, the use of EUROSTAT or TRACES database has no major influence regarding countries of origin and destination (Figs 2 and 3). However, the overall risk estimates (with EUROSTAT data the overall risk was twice as high) and months at higher risk, differed substantially between the two databases. Those differences need to be considered when comparing the outputs of this model with other models. The model presented here specifically addressed the qualitative (i.e. countries and months) and quantitative (amount of risk) variation of risk. This aspect may help policy makers to better interpret and apply models that use EUROSTAT or TRACES as source of information.

The model outputs indicate an annual probability for ASFV introduction by legal imports into EU of $5.21^{*}10^{-3}$, which approximately correspond to one outbreak each 192 years. Legal trade of animals always poses a risk; but in this case, the risk of ASFV introduction associated with live pig imports could be categorized as a low (but not negligible) compared with the risk associated with other pathways. In fact, results of recently published qualitative risk assessments for ASFV introduction into the EU, identified as the most risky pathway for ASFV introduction into EU the illegal imports of pigs and pig products from infected areas (EFSA, 2010; Gale et al., 2010; Wieland et al., 2011). It is important to highlight that other

pathways such as illegal import of pigs or pig products, movements of wild boar or introduction of fomites or ticks from infected areas, which may pose greater risks, were not considered in this study, mainly because of the lack of quantitative information. However, work is under way to complement the findings of the model presented here with qualitative or semi-quantitative risk assessments for other routes of ASFV introduction into the EU in order to assess the relative importance of the different risk pathways (under the ASFRISK EU project: EC, FP7-KBBE-2007-1, Project #211691).

In the current analysis, the country with the highest contribution to the risk of ASFV introduction into the EU is the Russian Federation (68% of total risk). This was to be expected, because among the source countries considered here, only Russia had reported recent ASF outbreaks, and furthermore, it had a relatively long period before detection of the first infected pig (29 days based on the WAHID database). Although imports of pigs and pig products are forbidden based on the OIE terrestrial code and EU legislation, since the first notification of the disease in Russian Federation (November 2007), this country was included in the analysis to quantify the risk associated with the HRP.

European countries that had the highest probabilities for ASFV introduction by legal imports of live pigs were Poland, followed by United Kingdom and Germany, mainly during the months of November and December. The higher risk in November and December can be explained by the seasonal high numbers of live pig imports coming from areas/countries with a (relatively) high probability of having an ASFV non-detected epidemic. It should be highlighted that, if geographical and cultural closeness between countries also results in relatively high levels of live pig legal imports, it may also be associated with increased levels of illegal pig and pig product imports or fomite movements, particularly if there are significant differences in pig prices. For example, in the case of Poland, which traditionally imports pigs from Russia, it is also an important exporter of live pigs to the RF (92 289 pigs exported from Poland to RF since 2004) (EUROSTAT database). This may increase the risk for ASFV introduction into EU, not associated with legal import of pigs, but to the vehicles returning from infected areas if they are not properly cleaned and disinfected.

In the potential situation that ASFV enters into one EU country, the potential risk for ASFV spreading to other countries during the high-risk period may be quite high, considering the high frequency of pig movements between EU countries. For example, in the case of a potential introduction into Poland, ASFV may easily spread to Hungary, Lithuania, Latvia and Romania, and from those countries, to other EU countries (EFSA, 2010; EUROSTAT database). This situation would result in severe economic consequences for the EU, because of the cost of control and eradication of the disease, as well as the resulting trade restrictions.

Sensitivity analysis on input parameters reveals that, in general, the model is robust. Changes in input values did not substantially modify the ranking of the countries and months at risk. However, the probability of survival to ASFV infection, the intraherd prevalence and the number of undetected outbreaks in the Russian Federation were the three parameters that had the strongest influence on model results. A 50% of increase in the base value of these three parameters resulted in an increase in the overall probability of ASFV introduction into EU by 51%, 48% and 34%, respectively. These results highlight the importance of using meaningful (i.e. realistic) input values for those three parameters, which could be obtained through good surveillance programmes as well as outbreak follow-up data from the current ASFV epidemic in the Russian Federation.

One of the most critical aspects of any risk assessment model is the capacity to incorporate reliable data and to consider the epidemiological aspects regarding the current situation of the disease under evaluation. In the work presented here, although there is scarce knowledge and literature published about the current situation of the ASF in the Transcaucasus region and Russian Federation, we have produced a flexible and easy to update tool that was illustrated with the best information available to us. For that reason, it would be extremely useful to carry out field studies to improve the knowledge about the epidemiological factors in the current affected areas to better understand the distribution and potential ASF spread to new territories. Such knowledge would allow improving the parameters/assumptions of this and other ASF risk-assessment frameworks, reducing the uncertainty of the results.

The methods and results obtained in this study will be useful for identifying the geographical areas and time periods at increased risk of ASFV introduction. Regular updating and improvement of this risk assessment model for the EU will provide support for the development of more effective risk-based surveillance and contingency plans and, ultimately, will help to reduce the risk of ASFV introduction into the EU.

Conclusion

This study presents the first quantitative risk assessment for ASFV introduction into the European Union for legal import of live pigs. Apart from the results presented here, it is shown that legal trade of live pigs does not pose a high risk for ASFV introduction into the EU, methods

and results obtained would help to allocate financial and personnel resources in geographical areas (Poland) and time periods (November) at increased risk, which ultimately will lead to a better prevention and control of potential incursions of ASFV into European Union and other free territories.

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Declaration of interest

None.

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RESEARCH ARTICLE Open Access

Risk of African swine fever introduction into the European Union through transport-associated routes: returning trucks and waste from international ships and planes

Lina Mur*, Beatriz Martínez-López and José Manuel Sánchez-Vizcaíno

Abstract

Background: The uncontrolled presence of African swine fever (ASF) in Russian Federation (RF) poses a serious risk to the whole European Union (EU) pig industry. Although trade of pigs and their products is banned since the official notification in June 2007, the potential introduction of ASF virus (ASFV) may occur by other routes, which are very frequent in ASF, and more difficult to control, such as contaminated waste or infected vehicles. This study was intended to estimate the risk of ASFV introduction into the EU through three types of transport routes: returning trucks, waste from international ships and waste from international planes, which will be referred here as transport-associated routes (TAR). Since no detailed and official information was available for these routes, a semi-quantitative model based on the weighted combination of risk factors was developed to estimate the risk of ASFV introduction by TAR. Relative weights for combination of different risk factors as well as validation of the model results were obtained by an expert opinion elicitation.

Results: Model results indicate that the relative risk for ASFV introduction through TAR in most of the EU countries (16) is low, although some countries, specifically Poland and Lithuania, concentrate high levels of risk, the returning trucks route being the analyzed TAR that currently poses the highest risk for ASFV introduction into the EU. The spatial distribution of the risk of ASFV introduction varies importantly between the analyzed introduction routes. Results also highlight the need to increase the awareness and precautions for ASF prevention, particularly ensuring truck disinfection, to minimize the potential risk of entrance into the EU.

Conclusions: This study presents the first assessment of ASF introduction into the EU through TAR. The innovative model developed here could be used in data scarce situations for estimating the relative risk associated to each EU country. This simple methodology provides a rapid and easy to interpret results on risk that may be used for a target and cost-effective allocation of resources to prevent disease introduction.

Keywords: African swine fever, Risk assessment, Transport, Trucks, Waste disposal

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Background

African swine fever (ASF) is one of the most devastating diseases of swine due to the high mortality caused (mainly in the hyper-acute and acute forms of the disease), the absence of effective vaccine, and the severe trade restrictions associated with its presence in the affected areas. ASF is caused by the infection of ASF virus (ASFV) which is transmitted by direct contact with fluids and excretions from infected animals. Blood, widely present in hyper-acute and acute forms of the disease, is considered the major route of direct transmission, containing high titers of virus for long lasting periods [1]. Other common ways of ASFV infection are the bites of infected ticks, and the indirect contact with various contaminated fomites and pig products, where ASFV remains infectious for long periods. As an example, ASFV infectivity persists more than 1000 days in frozen meat [2], 15 weeks in putrefied blood stored at room temperature [3] and one month in contaminated pig pens [4]. This long ASFV persistence explains that the introduction of contaminated pork meat or other pig products from international transports and, its eventual use to feed pigs, is one of the most frequents ways for ASFV introduction into free territories. For example, this route has been hypothesized to be the way of introduction in Europe, specifically in Portugal in 1957, in the Caribbean sea (Cuba) in 1971, in South America (Brazil) in 1978, Belgium in 1985 or, recently, in Georgia in 2007 [5].

Other fomites (e.g. vehicles, animal feed, veterinarians or contaminated material) have been also identified as important routes for pig diseases introduction/spread into free-territories [6]. In fact, returning trucks have been identified as one of the most important ways of spread of diseases such as classical swine fever (CSF) by studies performed in Denmark and The Netherlands [7,8]. Unfortunately, the risk associated to this route is not easy to be quantified, mainly because of the lack of detailed information and the need to estimate large number of parameters which increases the complexity and uncertainty of the models to be used. Some studies have assessed the introduction of some animal diseases through returning trucks in specific regions or countries [7,8] or through illegal meat [9]. Other were aimed to estimate the exposure of susceptible populations to swill or catering waste (e.g. [10-12]) in countries such as Denmark, The Netherlands, United States of America or United Kingdom. Even, recently published studies addressed the risk of ASFV introduction by imports of pigs [13]. However, to the best of the author´s knowledge, no studies have assessed the risk of ASFV introduction through transport-associated routes (TAR) in the whole European Union (EU).

After the introduction in Georgia in 2007, the spread of ASFV into Trans Caucasian countries (TCC) and Russian Federation (RF) territories has caused more than 200 notifications, with more than 120000 animals culled in the area [14]. Currently, ASFV spread is not controlled within RF, with continuous occurrence of outbreaks. Moreover, some of those outbreaks have been notified in very distant regions (>2500 km) from the initial outbreaks and are close (<150 km) to the EU borders [15]. In response to this situation, the EU authorities carried out some scientific studies to estimate the risk of ASFV introduction into the EU [16], which highlighted the need for the proper disinfection of returning livestock vehicles coming from affected areas [17].

Considering i) the severe sanitary and economic impact of ASF in the affected territories, ii) the current situation out of control in RF [15]; iii) the closeness of some ASFV outbreaks to the EU borders and iv) the evident concern of the EU authorities regarding the risk associated with returning trucks [17], it was considered valuable to estimate the potential risk of ASFV introduction into the EU by TAR. The semi-quantitative model presented here provides a simple and transparent method to interpret results, identifying the most critical TAR for each country and ranking the countries based on their relative risk for ASFV introduction.

Methods

The study presented here was aimed to estimate what the World Organization for Animal Health (OIE) guidelines of risk analysis referred to as “release or entry assessment” [18] for the three TAR analyzed. As we have no information and detailed data regarding the subsequent infection associated with contaminated trucks and with the potential illegal use of waste for animal feeding purposes for each of the 27 EU countries, exposure assessment was not evaluated in this work.

In collaboration with the Veterinary Epidemiology and Public Health group from the Royal Veterinary College (RVC) a semi-quantitative risk assessment model was developed to estimate the risk of ASFV introduction by the selected routes, using the combination of principles from the knowledge driven spatial models [19] and expert opinion elicitation methods previously used in other animal health risk assessments [20,21]. Risk factors related with the risk of ASFV introduction by each route were selected based on literature review. Information for these risk factors was gathered and further combined weighting their importance by expert opinion (EO) to obtain the relative risk for each country by each route. Consequently, this study was structured in three consecutive steps: (1) risk pathways definition, (2) likelihood estimation, and, (3) sensitivity analysis.

Risk pathways definition

The goal of the study was to estimate the spatial variation of the risk of ASFV introduction into the EU

countries by TAR and to identify the TAR at highest risk for ASFV entrance in each specific country. Based on historical data, current awareness and previous works [16] about ASF risk of introduction into EU, three TAR were considered in the model. The first one was referred as returning trucks and was intended to measure the probability of ASFV introduction into the EU by potentially contaminated livestock vehicles (i.e.. trucks) coming from affected areas. The second TAR was the waste disposal from international ships, which historically, has been one of the most important ways for ASFV introduction into free areas. The third TAR was the waste from international planes.

Likelihood estimation

An intensive literature review was performed to select and collect information about the risk factors that could be used to estimate the risk of ASFV introduction by each of the considered TAR pathways. Those risk factors, for which complete, reliable and updated data was available for all the EU countries, were selected for the model calculations. Sources of data, main assumptions and uncertainty associated to each of these risk factors have been summarized in Table 1.

The risk associated with returning trucks was estimated using three risk factors which were: (1) number of pig exports toASF-affected countries (i.e RF and TCC) (2) number of road border cross points with non EU countries (except borders with Switzerland, Lichtenstein and Norway) and (3) proportion of returning trucks not being properly disinfected. This last risk factor was defined using three different scenarios that allow the evaluation of the effect of preventive measures (i.e. high, medium and low percentage of truck disinfection) on the final risk values. For final calculations, medium scenario proportion of truck not properly disinfected was selected for all the countries.

In order to estimate the risk associated with waste disposal from international ships, we used the volume of goods and number of persons transported by different types of ships as an indicator of the volume of the potential contaminated products arriving by ships to the EU. For the purpose here, only movements coming from ASF-infected countries were considered for the analysis. Particularly, four different types of ship movements were considered, based on EUROSTAT official classification. The first type is cargo ships, which includes all type of ships for transporting goods. The second type is passenger ships, which includes ferries and other boats used for human transportation, excluding cruises. Short sea shipping (SSS) is the third type of ships, which includes movements of goods within small seas. Specifically here, only movements in Baltic and Black sea were considered, as they are surrounded by ASF infected countries. The fourth and final type of boat is cruises, particularly, only cruisers with at least one call in an ASF-infected country (African countries and RF) were considered in the analysis.

Similar to boat waste estimation, the number of commercial flights coming from ASF-affected areas was selected as risk factor for the volume of potential ASF-contaminated waste arriving by plane to the EU.

Risk factor standardization

Country data for each risk factor was obtained and further standardized into the same comparable scale to allow their comparison and further combination. Specifically, for each risk factor, data values of all the EU countries were transformed into six categories based on country data distribution. These categories are based on Natural Breaks of the data countries values for the specific risk factor, further adjusted using the Jenks optimization method of ArcGIS 9.3 (ESRI) [26]. For each RF, countries values were transformed into values from 0 to 5. For each category, a risk level was assigned, considering the following ranking: negligible (0); very low (>0 - ≤1); low (>1- ≤2); medium (>2 - ≤3); high (>3 - ≤4); very high (>4).

Expert opinion elicitation

Final results for each TAR were obtained by the weighted combination of the risk factor categories. To estimate the importance and consequent weight for each risk factor and route, an EO panel session was performed in Lisbon, September 23rd 2011, during the ASFRISK symposium. EO elicitation process have been widely used in many fields (engineering, sciences, social or medical research among others) to deal with uncertainties and gather information on parameters not formally described in literature. These methods are based on the opinions or judgments of experts in the specific field to be covered [27]. In this case, the group of experts included twenty-three international experts, with wide experience on animal health risk management, including three CVOs from EU countries, eight representatives from EU CVOs, and representatives from FAO, OIE, DG SANCO and Russian Agriculture Ministry, and highly experienced researchers in ASF.

The selected methodology for developing the EO sessions was a Delphi modified method, similarly to those used by Gale et al. and Gallagher et al. in previous works. Following basic steps of Delphi method, first of all, experts were given an introduction about the goal of the risk assessment, the structure and risk factors of the model, as well as the instructions for voting. Each expert was given an electronic remote control device associated with an interactive presentation. This system guarantees the anonymity of the votes and enables the on time

Table 1 Description of the risk factors used in the model for ASFV introduction into EU by TAR

Name	Risk factors	Parameter to be estimated	Data source	Assumptions	Uncertainty
P1	Number of live pigs exported from EU to ASF-affected countries by road	Number of potential ASF contaminated returning trucks	[22] (Nov. 2007–2009)	Only pig exports to TCC and RF were considered. It was assumed that trucks that export live pigs may enter into a farm and potentially become contaminated with ASFV.	Other type of trucks could also get in contact with ASFV in affected areas; however the most probable is that a pig truck enters into a farm.
P2	Number of the roads crossing EU national boundaries with non-EU states	Number of ways (and consequently, facility) of a truck to arrive by road to an EU country from non-EU countries.	[23]	Borders with all non-EU member states were included except members of the European Free Trade Association (Switzerland, Lichtenstein, Norway). It was assumed that higher number of cross border points, implies higher number of connections, and consequently easier to share trucks movements.	Other factors such as cultural relations, effectiveness and quality of controls or topography, were not considered.
P3	Three scenarios were used to approximate the proportion of returning trucks not properly disinfected	Returning trucks not properly disinfected	[7]	Despite disinfection of returning trucks from ASF affected areas is mandatory, this measure is not always 100% effective. For the best scenario, a 5% of returning trucks not properly disinfected was assumed, 15% for the medium scenario, and 25% for the worst case scenario.	As no field data is available related with efficiency of this measure, common scenarios were used for the 27 EU countries. If known, differences between countries may be simulated within the model by the selection of different scenarios in each country.
P4	Inward number of cargo ships from ASF-infected countries to EU ports	Potential ASF-contaminated waste introduced by cargo ships	[22]; [14]	Imports of goods were considered without differences between products. More volume of goods implies more waste.	Catering used in the cargo ship not necessary comes from the departure country.
P5	Inward number of passenger ships coming from ASF-infected countries to EU	Potential ASF-contaminated waste introduced by passenger ships (excluding cruises)	[22]; [14]	More passengers imply more catering and consequently, more waste.	Passenger ships not always have catering and do not imply that food comes from origin countries.
P6	Short sea shipping (SSS) ships coming from ASF-infected countries to EU	Potential ASF-contaminated waste introduced by SSS movements	[22]; GIS	Volume of goods transported by SSS movements by Baltic and Black sea. Only two sea regions were considered as potential risk for ASF introduction (Baltic and Black sea).	Higher volume of transported products not always implies higher number of crew on the boat and consequently higher volume of catering and food brought from origin countries.
P7	Proportion of cruise ships coming from ASF-affected areas by country	Potential ASF-contaminated waste introduced by cruises	$P7 = \frac{CA_i}{[C_p/p]}$	Assuming that these cruises bring catering food from departure or call countries.	A potential stop in an affected country does not always imply use of food from this country. Unknown origin of cruise catering increase uncertainty of this measure.

Table 1 Description of the risk factors used in the model for ASFV introduction into EU by TAR *(Continued)*

CA		Number of cruise ships arriving at EU ports after one stop in ASF-infected areas.	[24]	Assuming a similar number of cruises and origins in the different years.	Data from one year to another may change
Cp		Number of cruise passengers arriving at EU ports (Cp)	[22]		Data from one year to another may change
P		Average number of passenger by cruise ship was used to estimate number of cruisers (P)	[25]	Assuming a similar number of passengers by cruise.	Different types of cruises with different capacities could affect the final estimation
P8	Commercial passenger flights from ASF-infected countries to EU airports	Potential contaminated waste introduced by international passenger flights	[22];[14]	It was assumed that commercial flights from affected areas could potentially bring food from origin countries. The higher the number of flights from ASF-infected countries, the higher the risk of using ASF contaminated products.	Unknown origin of the catering increase uncertainty of this measure.

Detailed data about assumptions, uncertainties and data source of each risk factor used in the model was included in the table.

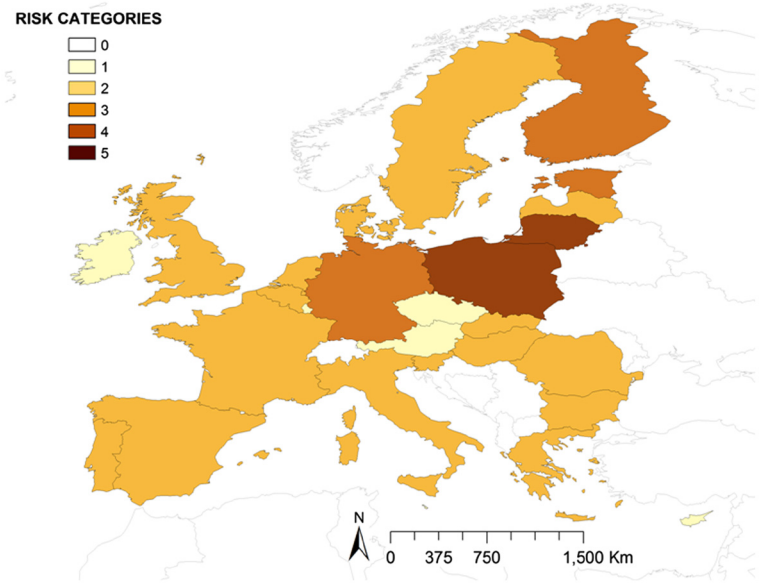

Figure 1 Distribution of the relative risk for ASFV introduction into the EU by transport-associated routes.

review of the votes, allowing the discussion and comments about them on the same session.

During the EO session, experts answered ten closed questions with a number of possible answers, for the comparison of the importance of each risk factor by route and within routes. The results of the EO were combined assigning equal weights to all the experts, which allows to obtain the weights by RF and route. These weights were computed using the percentage of votes that considered each RF/route as the highest risk for ASFV introduction.

Combining the results

Once all the weights were obtained by EO and each risk factor value was standardized, both were combined in order to obtain the relative risk of ASF introduction into the EU by TAR (RR_{TAR}). The way to combine these risk factor values (numerical values) was a linear weighted model, similar to those used in the knowledge driven models [19], using the weights obtained during the EO elicitation. The detailed explanation of this combination is described in the following formulas:

$$RRTAR = \sum_{i=1}^{n} [0.65(R.T) + 0.24(W.S) + 0.11(W.P)]$$

$$RRTAR = \sum_{i=1}^{n} \Big[0.65\ (0.33P1i + 0.33P2i + 0.33P3i) + 0.24\ (0.33P4i + 0.42P5i + 0.24P6i + 0.01P7i) + 0.11P8i\Big]$$

where n is the number of countries evaluated for ASF-TAR risk in the EU, *R.T.* is the risk value for returning

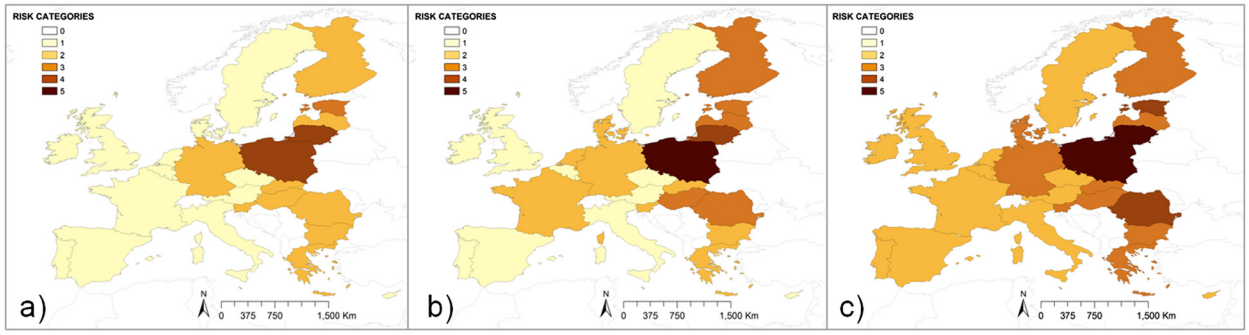

Figure 2 Relative risk of ASFV introduction by returning trucks when considering three different scenarios. Three different scenarios were used to approximate the proportion number of returning trucks not properly disinfected **a**) 5% (best scenario), **b**) 15% (medium scenario) and **c**) 25% (worst scenario).

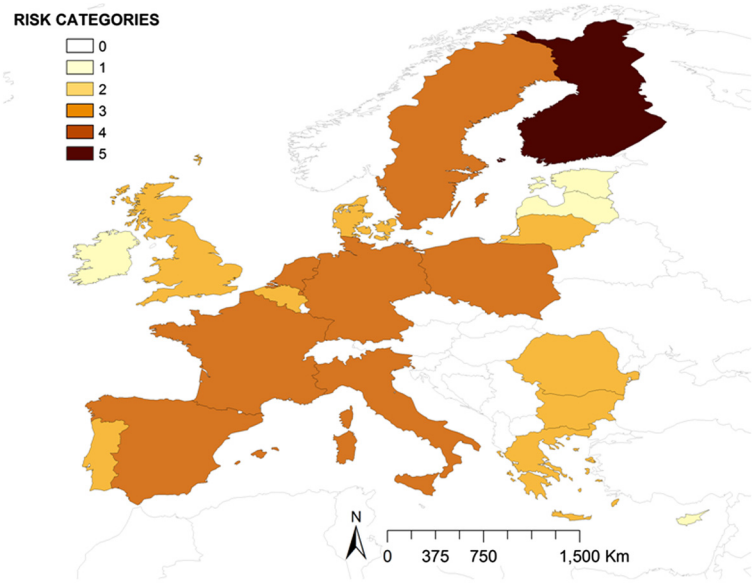

Figure 3 Relative risk of ASFV introduction into EU by waste from international ships.

trucks route and, *WS* and *WP* are the risk values for waste disposal from ships and planes, respectively. The second formula disaggregates the routes by risk factors used for its calculations, which definitions, data sources and uncertainties are explained in Table 1.

The final risk values were translated into qualitative results, following the same risk categories explained before, to facilitate the comprehension of the results.

Sensitivity analysis

All weights used in the model were analyzed in the sensitivity analysis (SA) by the 25% increase and decrease from their initial values, considering twenty different SA scenarios. Results obtained in each of these twenty SA scenarios were compared with the results obtained in the reference model (Figure 1). Correlation between results of these scenarios and the reference results was

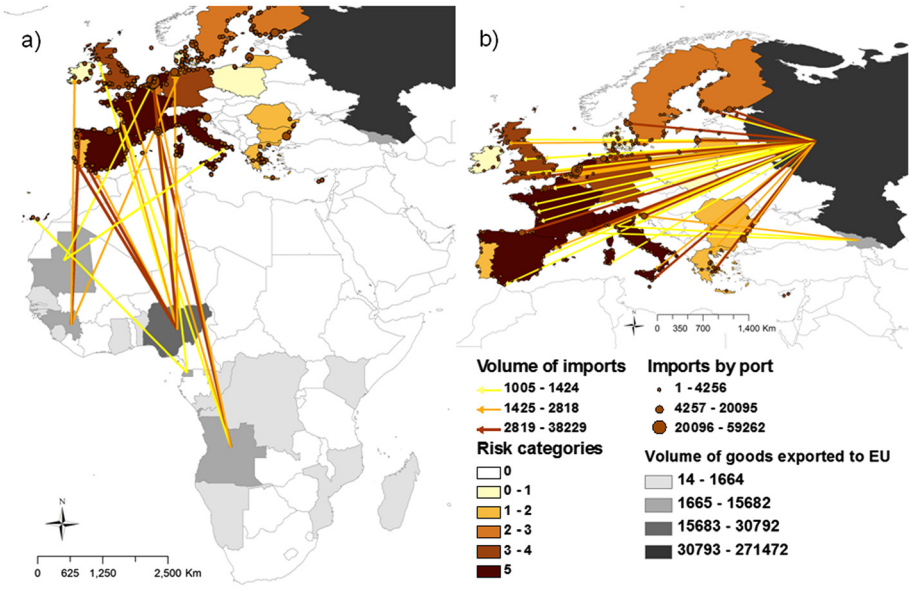

Figure 4 Relative risk of ASFV introduction into EU by waste from cargo ships. Results for the relative risk of ASFV introduction into EU by waste from cargo ships are represented with detail of the origin and destination of imports coming from Africa (**a**) and European ASF-infected countries (**b**).

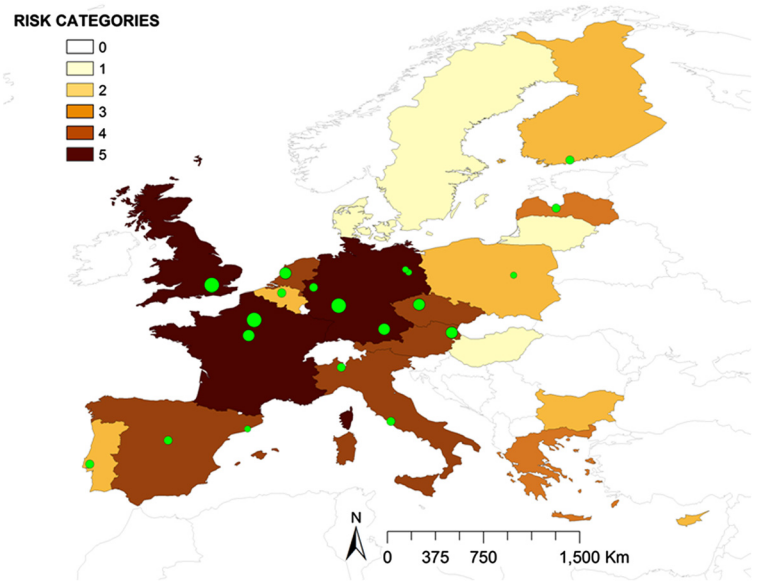

Figure 5 Relative risk of ASFV introduction into EU by waste from international flights. EU airports are shown in green dots graduated by the volume of extra-EU flights coming from ASF affected areas.

calculated by using the Spearman correlation coefficient (Rho) calculated with R-language (v. 2.14.1, R Foundation for Statistical Computing, Vienna, 2010). Additionally, the number of countries changing their risk category in the SA scenarios was represented.

All results were represented using choropleth maps with ArcGIS 9.3.1 (ESRI).

Results

Most of the EU countries (16 over 27) presented a low level of risk for ASFV introduction by TAR (RR_{TAR}). The minimum risk (0.53) was located in Malta and the maximum risk (3.52) in Poland. The countries at highest risk were Poland and Lithuania followed by Finland, Estonia and Germany, with medium risk (Figure 1).

The highest risk for ASF introduction was associated with returning trucks, accounting for the 65% of the total TAR risk. In the scenario with medium proportion of disinfected trucks, Poland had an estimated very high risk (Figure. 2) followed by Lithuania with high risk, and Estonia, Finland, Latvia and Romania, with medium risk, being all of them neighboring countries to current

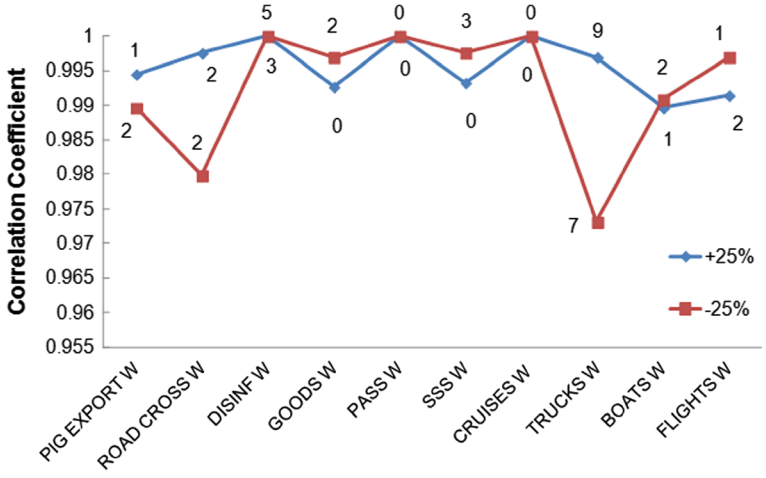

Figure 6 Correlation between sensitivity analysis scenarios changing risk factor weights (+ – 25%) and the reference model results. The number of countries that changed their risk category in the different scenarios is represented near the line.

affected area of RF. As it is shown in Figure 2, the level of disinfection of trucks meaningfully changes risk categories, from level two (low risk) in the case of Romania for the best-case scenario, to level four (high risk) in the worst-case scenario.

Waste from international ships was the second most relevant pathway in terms of risk. Risk map for this pathway (Figure 3) highlighted Finland with a very high risk, followed by several countries with a medium risk. Nevertheless, some countries concentrated very high risk of ASFV introduction associated to specific types of ships. For movements of passengers, considered as the most important type of ship movements by EO, only Finland and Poland registered movements from ASF affected areas, concentrating a very high and medium risk, respectively. In the case of cargo ships, the second most important boat type, four countries concentrated a very high level of risk (Figure 4). The detailed analysis of this type of movements revealed that the main origin of these cargo ships movements was RF, followed by Nigeria and Angola, however different patterns of risk were found among destination countries. For SSS movements, four countries surrounding the Baltic sea (Denmark, Finland, Lithuania and Sweden) as well as Bulgaria in the Black sea, concentrated very high levels of risk. For cruise movements only five North European countries concentrated very high risk levels, although the relative importance of this type of movements was considered low by EO.

Finally, waste from international planes was the pathway posing highest risk for United Kingdom, France and Germany (Figure 5). The main EU airports are represented in Figure 5 using graduated symbols based on the number of extra-EU commercial flights received from ASF-affected countries.

SA revealed that the model is robust. The lowest correlation coefficient obtained between the reference scenario and each of the different SA scenarios was Rho = 0.97 ($p < 0.01$) (Figure 6). Analyzing the impact that changes in inputs had on the country risk category, none of the countries experienced a change greater than one category. All these findings revealed that substantial changes of 25% in the initial weight values do not meaningfully affect final risk results.

Discussion

This is the first study aimed to assess the risk of ASFV introduction into the EU associated to TAR. Current presence of the disease without control in areas of RF close to the EU borders, together with results of the EFSA advise [16], other studies [28] and recent published legislation [17], point out the importance that illegal trade and other potential pathways such as transport fomites may have in the ASFV introduction into the EU, which remarks the importance of the study here.

However, the estimation of TAR is not a simple task. Vehicles and waste from international means of transport have been frequently suggested as a potential route for disease spread, specifically for ASFV introduction into free areas [29,30]; but no studies have quantified this risk. The lack of information for these TAR does not allow to use traditional risk assessment models making necessary to develop alternative approaches to analyze the risk of ASFV introduction by these pathways. The methodology proposed here combines methods used in the knowledge driven models used for spatial modeling of diseases [19] as well as methods for the risk estimation based on expert opinion elicitation [20,21]. Moreover, we used available data on risk factors, conveniently standardized, weighted by EO and linearly combined (as done in spatial modeling), to estimate the relative risk of TAR in the different EU countries. Although results should be cautiously interpreted considering all the assumptions and uncertainties associated with the model structure and data used, the approach is believed to be useful to evaluate the TAR risk. This study was specifically performed considering the selected routes of entrance, and, importantly, the specific characteristics of the pathogen, for the risk tended to be estimated. For example, the long survival of ASFV in all kind of meat and infected products allows to measure the risk based on potential incoming volumes of infected material, without considering the survival time of the virus on it. However, when adapting the methodology presented here for other animal diseases or routes of entrance, this important feature, as well as many other specific characteristics and parameters, should be modified conveniently to incorporate the features of the disease under study.

One of the most important aspects to be considered is that the model does not provide probabilities, but compares the relative risk between the 27 EU countries based on the risk factors evaluated. Indeed, a high value on the model results does not imply an absolute high level of risk, but a higher one compared with other EU members. On the other hand, the selection of information for each of the risk factors used in the model is influenced by the quality and availability of data sources for the 27 EU countries. For example, the degree of cleaning of returning trucks is based on scenario rather than real data due to the absence of this data for each of the EU countries. Therefore the results presented here depend on the quality and reliability of this data. It is important to consider also that the model only estimates the risk of entrance/release of potential ASFV-contaminated material/transports, but does not consider the subsequent exposure of the susceptible livestock population in the destination country.

Another idiosyncrasy of the model is the use of weights obtained by EO for combination of risk factor and pathways. EO process is a valuable method widely use when no other "more objective" information is available (i.e. literature, etc.) and particularly for the estimation of complex parameters or parameters with significant uncertainties, as some of the presented in this study. Particularly, Delphi approach is one of the most frequently used methods of EO that originally does not allow for interaction between the experts [27]. However, in this study small modifications were made by the little interaction between experts during the results presentation and the use of electronic devices for voting. This technique implies many advantages, being an adequate way to collect information for solving problems. However, the lack of universal guidelines or standardized procedures for its performance could arrive into difficulties that should be cautiously considered [31]. Some problems of the technique could be derived from the inappropriate selection of the experts, the lack of previous information, the inadequate performance of the questionnaire or the combination of the results. Nevertheless, this method may provide a more realistic and updated view of the scenarios under evaluation, in this case related with ASF risk, based on the experts valuable experience. Moreover, and because weights used in the weighted combination of the risk factor are a critical aspect of the model, an intensive SA was performed in order to identify the impact of these estimated weights in the final results. This SA reveals that the model is robust and do not significantly change when changing the weights provided by EO. In fact, none of the countries changed more than one category in the different SA scenarios evaluated. For example, the 25% decrease on the weight of returning trucks, which is the scenario with lowest correlation coefficient (Rho = 0.97), affected categories of seven countries. Most of them (three countries) changed from low to very low risk, two decreased from medium to low risk, and two from high to medium risk. These changes result in a very similar risk map, but with a slight difference of risk category in these countries, which confirms the robustness of the model.

At the same time, the use of different scenarios in some of the measured parameters allows Animal Health (AH) Authorities in each EU country to have the possibility to select the scenario that more realistically represents their current situation based on their expert opinion. For example, we are providing three different results based on certain assumptions, but AH Authorities may consider that for their countries only the scenario one is realistic, so they will have the possibility to select it and visualize the correspondent outputs. This flexibility, as well as, the easy update and the possibility of incorporation of more detailed information (if available for some countries) instead of being considered a limitation, is considered as one of the main strengths of the model.

Model results reveal that the median of the risk values for ASFV by TAR in the 27 EU countries is low (for 16 of the 27 EU countries), although big differences were found between countries and pathways. An expected result of the model is that EU countries closer to RF and TCC borders are the ones at higher risk for ASFV introduction by TAR, being Lithuania and Poland the countries at higher risk for ASFV entrance, followed by Finland, Estonia and Germany. Returning trucks is the TAR at highest risk for ASFV introduction into the UE, being almost three and six times more important than waste from ships and planes, respectively. This result is in agreement with the EU commission risk perception which recently approved a legislation [17] that strengthen and remind the importance of cleaning and disinfection for returning livestock vehicles coming from affected areas. In fact, the differences found in the results of the three scenarios for the disinfection of trucks (Figure 2) highlight the importance of that measure in preventing the entrance of animal diseases into free territories. On the other hand, ships waste has two times higher risk than plane waste. Ships waste has been recently suggested as the origin of the outbreaks in the Caucasus region [5], which may have influenced the opinion of the experts regarding their weights. Again we should highlight that the risk associated to ships and planes waste would depend not only on the release in the EU country, but on the final exposure, or contact, with susceptible populations, and this fact has not been measured on this work.

The analysis in detail of the results obtained for the different countries and the different analyzed pathways give us a better characterization of the risk. For example, in the case of Lithuania, although it has an overall high risk of ASF introduction by TAR, this risk is mostly associated to trucks, but not to waste from international ships or planes. These results are certainly influenced by the geographic location of the country, close to the current affected area, and the intense commercial relations with RF, which has been demonstrated by the amount of pig exports to this country. The opposite case is Germany that resulted in a medium risk for the overall TAR, but only a very low level for returning trucks. In this case the presence of most of the EU airports (50%, five over ten) that receive large number of flights from ASF-affected countries [22] determines the high level of risk associated to waste from planes. Similarly, Germany is a very important country in maritime trade (cargo ships, SSS and cruises) which explains also the high risk associated to waste from ships. Other countries with high risk associated to waste from planes are France and United Kingdom, where the two most

important airports in terms of number of flights coming from ASF-affected countries (Charles de Gaulle and Heathrow, respectively) are located (Figure 5).

Another interesting result is related with the big differences found among the different ship types. Although Finland is the unique country that concentrates an average very high risk for waste from ships pathway, other countries are only highlighted when a specific type of ship movements is analyzed. For example, Bulgaria has an estimated very high risk by SSS movements through Black sea, particularly associated with the port of Burgas, the second most important port in the Black sea [32]. Several countries surrounding the Baltic sea (Denmark, Finland, Lithuania or Sweden among others) are also highlighted in SSS movements and cruises, mainly due to their geographical closeness and trade with RF.

However, the most interesting results are related with waste from cargo ships, for which, four countries concentrate very high risk (France, Italy, the Netherlands and Spain). The detailed analysis of these movements, considering origin and EU destination ports, reveals some interesting differences among these countries. For example, in Netherlands, the risk both from RF as well as from Africa (mainly from Angola and Nigeria) is particularly concentrated in the port of Rotterdam, the one that receives the biggest amount of potentially risky cargo ships (those coming from ASF-affected countries) in the EU. However, in Spain the risk is more distributed, with two important ports receiving high number of cargo ships from Africa and other two from RF. In this particular case, these countries with the same level of risk present different profiles with one or several important ports in terms of risk. This fact enhances the importance of the detailed analysis of these results (represented in Figures 2, 3, 4 and 5) that could be much more informative to the policy makers than the general overview of the results (Figure 1).

Authors believe that this model has an important logical and biological approach as its results reflect areas and pathways identified at high risk by experts. This kind of models built using a simple and easy to understand methodology, are faster to develop and easier to interpret compared with the quantitative ones, and are particularly suitable when few information is available. For this reason this model may be considered as an adequate alternative in data scarce situations to provide a scientific support to risk managers, and ultimately, to prevent animal diseases introduction in free territories.

Conclusions

We developed a semi-quantitative new risk assessment approach to estimate the relative risk for ASFV into each EU country associated with TAR. The absence of available data for this pathway and the complexity of the estimations due to the huge uncertainties associated, lead us to develop a semi-quantitative model based on the use of risk factors as risk estimators. The results of the model indicate that the median risk of ASF introduction in EU countries is low and mainly associated to returning trucks, although some countries concentrate higher levels of risk such as Poland and Lithuania. Methods and results of this study may help to allocate surveillance and other risk reduction measures to prevent or minimize the potential impact of ASF introduction into the EU.

Competing interests

The authors declare that they have no competing interests.

Author's contribution

LM, BML and JMSV designed the model structure, the expert opinion session and decided the methodology to be used in the study. LM performed the data collection and model development. BML and JMSV critically reviewed the model results and suggested changes and adaptations to improve the quality of the study. All authors participated in drafting the manuscript, and have read and approved the final manuscript.

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Modular framework to assess the risk of African swine fever virus entry into the European Union

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Abstract

Background

The recent occurrence and spread of African swine fever (ASF) in Eastern Europe is perceived as a serious risk for the pig industry in the European Union (EU). In order to estimate the potential risk of ASF virus (ASFV) entering the EU, several pathways of introduction were previously assessed separately. The present work aimed to integrate five of these assessments (legal imports of pigs, legal imports of products, illegal imports of products, fomites associated with transport and wild boar movements) into a modular tool that facilitates the visualization and comprehension of the relative risk of ASFV introduction into the EU by each analyzed pathway.

Results

The framework's results indicate that 48% of EU countries are at relatively high risk (risk score 4 or 5 out of 5) for ASFV entry for at least one analyzed pathway. Four of these countries obtained the maximum risk score for one pathway: Bulgaria for legally imported products during the high risk period (HRP); Finland for wild boar; Slovenia and Sweden for legally imported pigs during the HRP. Distribution of risk considerably differed from one pathway to another; for some pathways, the risk was concentrated in a few countries (e.g., transport fomites), whereas other pathways incurred a high risk for 4 or 5 countries (legal pigs, illegal imports and wild boar).

Conclusions

The modular framework, developed to estimate the risk of ASFV entry into the EU, is available in a public domain, and is a transparent, easy-to-interpret tool that can be updated and adapted if required. The model's results determine the EU countries at higher risk for each ASFV introduction route, and provide a useful basis to develop a global coordinated program to improve ASFV prevention in the EU.

Keywords

African swine fever, Emerging disease, Introduction, European Union, Pigs, Risk assessment, Semi-quantitative framework, Transboundary disease

Background

The European Union (EU) has an ever-increasingly highly industrialized and specialized pig production sector [1], and is the second largest pig producer in the world with 22.6 million tons of pork produced in 2012 [2]. To maintain this high level of production and the current swine health status, it is crucial to prevent the introduction and re-introduction of infectious diseases, particularly OIE notifiable diseases such as African swine fever (ASF). The

introduction of ASF into any EU country would result in an immediate export ban of pigs and pig products from the infected area. This could cause huge losses for the affected country, especially given the ban of intra-EU movements as it poses the highest volume of trade, but also to the EU as a whole. The EU is a net exporter of pork, with 2.3 million tons of pork exported to third countries in 2012. Furthermore, there is the potential to increase exports due to high demand from Russia and China [2], besides important intra-EU trade.

ASF is one of the most devastating swine diseases given its high mortality, economic losses as a result of trade restrictions, and the fact that no vaccine is available for its control [3]. Traditionally, this viral infectious disease has been widespread on the African continent, where it still remains in many countries. In 1960, ASF spread to some southern European countries (Portugal and Spain), where it persisted for more than 30 years [4]. On the island of Sardinia (Italy), ASF has been endemic since 1978, which is the cause of much concern, although it has not spread to other EU areas [5]. In other European countries and some territories of the American continent (e.g., Brazil [6], the Dominican Republic, Haiti and Cuba [7]), ASF virus (ASFV) was also introduced in the 20th century, but was swiftly eradicated by drastic control programs.

Since its introduction into Georgia in 2007 [8], ASFV has spread to Russia, Armenia, Azerbaijan, and later to the Ukraine and Belarus, and has affected both domestic pig and wild boar (*Sus scrofa* L.) populations. More recently, in January and February 2014, ASF was confirmed in dead wild boars in Lithuania and Poland [9]. This situation is perceived to be a major threat for European pig producers and has increased disease awareness among Eastern EU member states [10,11].

Since there is no vaccine available for ASFV, disease prevention and rapid control require safe disposal of waste from international ports and airports, effective surveillance, disease awareness in high-risk areas, and regularly updated control and contingency plans. The design of tailored prevention and control strategies benefits from knowledge of the identification and allocation of the risks for ASFV introduction, which can be generated transparently through risk assessment [12].

Various EU countries have already made country-specific risk assessments. Finland has analyzed the potential routes of ASFV introduction by emphasizing the risk of wild boar infection in the country, as well as the importance of biosecurity measures to prevent domestic pigs coming into contact with ASFV [13]. Germany has analyzed the risk associated with returning animal transport vehicles, which it has classified as moderate for the transport of breeding pigs, but as high for movements of pigs for fattening and slaughter [14]. Other analyses have been carried out to estimate the risk of ASFV introduction into the United Kingdom [15], Poland [16] and Denmark [17]. However, none has been published in the peer-reviewed scientific literature. Consequently, the results are available only upon request to the authors.

The risk of ASFV entry into the EU has been analyzed by the authors of the present study for five different pathways, these being: i) legal imports of live pigs during the high-risk period (HRP; i.e. before detecting the first case in the exporting country) [18]; ii) legal imports of different types of pig products also during the HRP [19]; iii) illegal imports of pig products [20]; iv) transport fomites (including contaminated trucks or waste from international planes and ships) [21]; and v) wild boar movements [22]. The objective of the present work was to integrate all these pathways into a flexible and transparent modular framework to enable the

visualization and comprehension of the risk of ASFV introduction into EU by these five different pathways. The results of this framework will identify the EU countries at higher risk for all the analyzed pathways, in which specific control strategies can be adopted to prevent ASFV entry into the EU. In addition, the provided structure can be later used as an example to assess the risk of ASF entrance into other regions or can be conveniently adapted for the introduction of other diseases.

Methods

Model structure

A modular risk assessment framework was developed to separately integrate the risk assessments for the five main routes of entry (Figure 1) in order to provide an overview of the risk of ASF introduction into the EU. Five modules were developed to estimate the risk of ASFV entry into 27 of the 28 EU countries[a] via each analyzed pathway: legal imports of live pigs, legal imports of pig products, illegal imports of pig products, transport-associated fomites, and wild boar movements. The risk pathways included in the analysis were based on a literature review that considers ASFV transmission mechanisms, known routes of introduction into previously free areas, and the current epidemiological situation.

Figure 1 Detailed structure of the modular framework. The five risk pathway modules are represented and include the main steps of the respective quantitative and semi-quantitative models. P (Probability in the quantitative assessments, Proxy in the semi-quantitative assessments), RV (Risk Value), JENKS NB (Jenks Natural Breaks), WLC (Weighted linear combination of values), Manual NB (Manual Natural Breaks), X (Multiplication of values) + (sum of values).

All five modules, and consequently the modular framework that integrates them, correspond to the entry assessment component as defined in the OIE risk analysis framework, where entry addresses the likelihood of a commodity infected by a certain pathogen (hazard) being released into a particular territory [12]. The exposure and consequence assessments, which have been previously published [23], were not included.

Description of pathway modules

Different approaches were taken to develop the five risk assessment modules after considering pathway-specific characteristics and data availability. The risk of entry via legal import pathways was estimated by quantitative models, whereas risk was assessed by semi-quantitative models for illegal imports, wild boar and transport-associated fomites.

The legal import (live pigs and pig products) risk assessments provided risk estimates as probability distributions for ASFV introduction. In order to facilitate the visualization and interpretation of these results, the mean probabilities of ASFV introduction were categorized into six risk scores (risk scores from 0 to 5) based on natural breaks calculated by Jenks algorithm [24]. All the semi-quantitative models followed a similar approach using proxy indicators (parameters related to the level of risk and for which enough data are available). The data values for each proxy indicator were categorized using natural breaks (by Jenks algorithm for transport-associated fomites and wild boar, and manual natural breaks for the illegal pathway), and were consequently converted into six scores (scores from 0 to 5). Each

module produced an overall risk score for all the EU member states, calculated by a weighted linear combination of the scores of the proxy indicators. The weights of each parameter were obtained by expert opinion elicitation. The use of such methodology produces risk scores from 0 to 5 that reflect probabilities of 0 = negligible, 1 = very low, 2 = low, 3 = medium, 4 = high and 5 = very high to compare the remaining countries within the same pathway.

The detailed structure, parameters, inputs and outputs for these modules are available in separate publications [18-22]. The present work provides only a brief summary of each module in order to facilitate the comprehension of the adjustments made and, consequently, the proper interpretation of the results obtained.

Module 1 and 2: Legal imports.

For both legal import pathways (i.e., live pigs and pig products), two quantitative risk assessments [18,19] were conducted as detailed data were available for not only the frequencies and amounts of imports of pigs and products from extra-EU countries (considered to be countries of origin, or "country o") to EU member states (countries of destination "d"), but also for the numbers of pigs and pork production quantities in the countries of origin [25]. For both modules, a similar structure based on a scenario tree was used (Figure 2). For pig products, three different types of pork products, referred to as fresh meat (referred to as "a"), frozen meat (referred to as "b") and processed products (salted, smoked or fat products) (referred to as "c"), were considered to account for the different ASFV survival periods for these products [19]. The models were developed in @ Risk version 5.5 (Palisade Corporation, Newfield, NY, USA).

Figure 2 Scenario tree for the legal import of pigs and legal imports of products pathways.

Module 3: Illegal imports of pig products

As data on illegal imports are very scarce and the probabilities associated with most steps of the risk pathway would be difficult to estimate quantitatively, a different approach was adopted. Illegal importation of pig products for personal consumption and for commercial purposes was modeled semi-quantitatively using ten social, economic and geographical factors, also called proxy indicators, which were assumed to influence the risk of illegal import (more information is available in Table 1). Further details on the illegal import model and its results are available in [20].

Table 1 Parameters and sources of data employed in the modular framework for the ASF risk assessment

Pathway	Parameter	Definition	Source
L.PIGS &L.PROD	Po	Probability of infection in the country of origin (country o)	Beta(α_1, α_2) α_1 = X + 1; α2 = M-(X + 1) X:number of outbreaks by month[26] M: number of months considered
	Ou	Number of undetected outbreaks before official notification in country o	[9]
	To	Average herd size in country o	To = No/So
	No	Pig population in country o	[9,27]
	So	Pig establishments in country o	[27,28]
	Hp	Intra-herd prevalence	[29,30]
	Ps	Probability of an ASF-infected pig surviving infection	[31]
L.PIGS	P1s	Probability of selecting an ASF-infected pig from country o in month m	Beta (α_1, α_2) α_1 = NI + 1; α2 = No-(NI + 1) NI = Po x Ou x To x Hp;
	Pt	Probability of survival during transportation	[32]
	$P2_S$	Probability of a pig surviving	$P2_S$ = Ps*Pt
	Sn^{odm}	Imports of live swine (number of pigs) from country of origin (o) to the EU destination country (d) in month m (in the last 5 years). In order to transform Eurostat imports data (in 100 kg) into number of pigs, a standard weight of 100 kg was assumed per pig.	[25] Normal (μ, σ)
	Sp_{odm}	Probability of an ASF infected animal from country o entering country d in month (m)	Binomial (n, p) n = Sn^{odm} p = $P1_S$ x $P2_S$
	Pf_S	Probability of having at least one introduction of ASFV into one EU country (d) from one of country of origin (o) in month m by legal imports of live pigs	$Pfs = 1 - \prod_{odm}(1 - sp_{odm})$
	Pm	Probability of a pig being grown for meat production	Normal(Mo/No)
L. PRODS	Mo	Number of slaughtered pigs in countries o	[27] (2005/2009)
	PC	kg of meat obtained per slaughtered pig (per 100 kg)	[27] (2000-2009)
	Co	Annual pig meat production per country (100 kg)	[27] (2005-2008)
	$P1_p$	Probability of selecting infected ASFV pig meat from country o in month m	P1p: Mi / (Co/12), Mi: Po*Ou*To*Hp*Ps*Pm*PC
	$P2_p$	Probability of meat belonging to one of the different types of products considered	[25] (2008-2009)
	$P3_p$	Probability of ASF virus survival in each meat product type during transport.	[33,34]
	pn^{odm}	Imports of each pig meat product type (100 kg weight) from country “o” to EU country “d” in month “m”	[25] (2005-2009)
	Pp_{odm}	Probabilities of ASF infected pig products of different types (a-c) from country o entering country d in month m	Binomial (n, p) n = pn^{odm} p = $P1_p$* $P2_p$ * $P3_p$
	Pf_p	Probabilities of having at least one introduction of ASFV into one EU country (d) from one country (o) in month m by legal imports of each pig products type (a-c).	$Pfp = 1 - \prod_{odm}(1 - Pp_{odm})$

	PT_P	Probabilities of having at least one introduction of ASFV into one EU country (d) from one country o in month m by legal imports of any pig product type.	$PTp = 1 - \prod_{i=a}^{c} (1 - Pf_p)$
ILLEGAL	$P1_I$	Probability of release through illegal importation for personal consumption	Sum of weighted risk scores for P3, P4 and P5
	$P3_I$	Outbound tourism to ASF-affected countries. Holiday or business trips of 1 night or more from EC27 to Africa and Russia, arrivals of non resident visitors at national borders of Georgia	[25], Georgian National Tourism Agency
	$P4_I$	Inbound tourism from ASF-affected countries: Arrivals to EU27 of non residents from Africa and Russia staying in hotels, etc.	[25]
	$P5_I$	Residents (citizens)from ASF-affected countries	[25]
	$P2_I$	Probability of release through illegal importation for commercial purposes	Sum of weighted risk scores for P5, P6 and P7
	$P6_I$	Price of pork. 2010 annual average price of Grade E carcasses (55-59% lean meat percentage) in euros per 100 kg	European community
	$P7_I$	Geographic position	Sum of weighted risk scores (P8,P9,P10)
	$P8_I$	Number of ports and airports	World Port Index 2009; [25]
	$P9_I$	Distance in km to nearest ASF-affected country (from country border to border of nearest ASF-affected country)	Shapefile of national boundaries
	$P10_I$	Number of international terrestrial border points with non EU member states	FAO Geonetwork: shapefiles of railways, roads and waterways of the World VMAP)
TAF	P1t	Number of potential ASF-contaminated returning trucks. Number of live pigs exported from EU to ASF-affected countries by road	[25] (Nov. 2007-2009)- (ComExt)
	P2t	Number of ways (and consequently, facility) of a truck to arrive by road in an EU country from non EU countries. Number of roads crossing EU national boundaries with non EU states	FAO Geonetwork. Roads of the World
	P3t	Probability of returning trucks not being properly disinfected	[35]
	P4t	Potential ASF-contaminated waste introduced by cargo ships. Inward number of cargo ships from ASF-infected countries to EU ports	[25]-Traditional international trade database access (ComExt); [9]
	P5t	Potential ASF-contaminated waste introduced by passenger ships (excluding cruises). Inward number of passenger ships from ASF-infected countries to the EU	[25]-Traditional international trade database access (ComExt); [9]
	P6t	Potential ASF-contaminated waste introduced by Short sea shipping (SSS) movements. Ships from ASF-infected countries to the EU	[25]-Traditional international trade database access (ComExt); GIS
	P7t	Potential ASF-contaminated waste introduced by cruises. Proportion of cruise ships from ASF-affected areas per country	$P7t = \frac{CAi}{[Cp/p]}$
	CA	Number of cruise ships arriving at EU ports after one stop in ASF-infected areas	Travelocity. http://travel.travelocity.com
	Cp	Number of cruise passengers arriving at EU ports (Cp)	[25] (ComExt)
	p	Average number of passengers per cruise ship	Truecruises. http://www.truecruise.com/cruise-ship-database.aspx
	P8t	Potential contaminated waste introduced by international passenger flights. Commercial passenger flights from ASF-infected countries to EU airports	[25; 9]
WB	$P1_W$	Probability of wild boar becoming infected in country o through contact with infected wild boar	$P1_w = P4_w * P5_w$
	$P2_W$	Probability of wild boar becoming infected in country o through contact with infected domestic pigs	$P2_w = P6_w * P7_w$
	$P3_W$	Probability of infected wild boar crossing national border	$P3a_w$: $P8_w * P9_w$ $P3b_w$: $P9_w * P10_w$
	$P4_W$	Wild boar outbreak density in countries o	[9] 2007-2012
	$P5_W$	Wild boar population density in countries o	[36-38]
	$P6_W$	Density of domestic pig outbreaks in countries o	[9] 2007-2012
	$P7_W$	Domestic pig population density in countries o	[36]
	$P8_W$	Surface of shared wild boar suitable habitat along national borders	Corine land cover
	$P9_W$	Distance from EU countries to the nearest outbreak (wild boar)	[9] 2007-2012

$P10_W$	Distance from EU countries to the nearest outbreak (domestic pig)	[9] 2007-2012

L.PIGS (Legal imports of pigs), L.PROD (Legal imports of products), ILLEGAL (Illegal imports), TAF (Transport associated fomites) and WB (wild boar).

Module 4: Transport fomites

Similarly to the model for illegal imports, a proxy-based semi-quantitative model was used to estimate the risk of introduction through fomites associated with transport vehicles. The three routes that considered transport-associated fomites were: trucks returning from ASF-affected areas, waste from different ship types, and waste from international planes [21].

Module 5: Wild boar movement

The risk of ASFV introduction through wild boar (*Sus scrofa L.*) movements was estimated for those EU countries sharing borders with non EU countries from Eastern Europe, considered to be the origin of the risk. For this purpose, a semi-quantitative model was employed that considered two sources of infection: wild boar infection in the country of origin through contact with ASF-infected wildlife (wild, feral or captive) or through contact with ASF-infected domestic pigs. Afterward, the introduction of ASFV into the EU was assumed to occur through the natural movement of infected wild boars or through contacts of infected wild boars with other wild boars. The detailed structure and parameters considered in the model are described in Table 1 and elsewhere [22].

Overall risk of entry

The modular framework was implemented in Microsoft Excel (Microsoft Office 2010 Professional Edition) and is available as an additional file (Additional file 1). All the modules summarized in Section 2.2 were included with a first worksheet that documented the methodology, module structure, assumptions and limitations, and a second worksheet with all the data inputs and calculations employed. A summary table with all the information (definitions and data sources) of all the parameters used in the framework is presented in Table 1. Additionally, the final risk scores of each country by all analyzed pathways were summarized in Table 2.

Table 2 Risk scores of the five modules for the 27 EU state members

Country	Legal Pigs	Legal Products	Illegal	Transport	Wild boar
Austria	0	0	2	1	NS
Belgium	1	0	1	2	NS
Bulgaria	0	5	2	2	2
Cyprus	0	0	2	1	NS
Czech Republic	0	0	2	1	NS
Denmark	1	1	1	2	NS
Estonia	2	0	2	3	3
Finland	4	0	2	3	5
France	4	3	4	2	NS
Germany	3	4	4	3	NS
Greece	4	0	2	2	1
Hungary	0	0	1	2	2
Ireland	2	2	1	1	NS
Italy	0	2	4	2	NS
Latvia	0	0	2	2	4
Lithuania	0	0	2	4	3
Luxembourg	0	0	1	1	NS
Malta	0	0	1	1	NS
Netherlands	0	1	2	2	NS
Poland	3	1	2	4	4
Portugal	0	0	2	2	NS
Romania	0	4	2	2	4
Slovakia	0	0	1	2	2
Slovenia	5	0	1	2	NS
Spain	0	1	3	2	NS
Sweden	5	0	2	2	NS
United Kingdom	3	3	4	2	NS

(NS: not studied). Risk scores equal or higher than 4 were highlighted using boldface numbers.

Sensitivity analysis

For each module, sensitivity analyses were previously conducted to assess the robustness of the model and the influence of the different input parameters and/or the weights assigned to the proxies on the model results [18-22]. For the modular framework, the influence of the method used in each module to categorize the data of the proxies and risk scores was also assessed. In the individual risk assessments, categorization into risk scores was based on the natural breaks calculated with Jenks algorithm [24], except for the illegal pathway module. In the illegal pathway module, the parameter values tended to be clustered at lower values with a few extreme high values. Thus, Jenks algorithm led to quite low values which fell into relatively high risk scores. Therefore, a manual definition of the natural categories was preferred.

In the sensitivity analysis, other commonly used categorizing methods, specifically quantiles and geometric intervals, and Jenks algorithm for the illegal pathway, were used to calculate the risk scores for all five pathways. The results obtained with these alternative categorization methods were compared with the original method. Specifically, the list of countries at higher risk (a risk score equal to 4 and/or 5) for each pathway when using the different categorization methods is presented in descending order (from highest to lowest) in Table 3. Variations in the prioritization order, and the number of countries at higher risk, are also included in this table.

Table 3 Ordered list of country at highest risk per pathway using different categorization methods (NB: Jenks Natural Breaks, Q: Quantiles, GI: Geometric Interval; MNB: Manual Natural Breaks; RS: Risk Score)

Pathway	Categorization method	Countries with RS 5	Countries with RS 4	Countries that underwent changes in risk order	New countries with RS 5	New countries with RS 4
Legal pigs	**NB**	Sweden > Slovenia	Finland > Greece > France			
	Q	Sweden > Slovenia	Finland > Greece > France > Poland	0	0	1
	GI	Sweden > Slovenia	Finland > Greece > France > Poland > UK > Germany	0	0	4
Legal products	**NB**	Bulgaria	Romania > Germany			
	Q	Bulgaria > Romania > Germany > UK > France	Ireland > Italy > Netherlands > Spain	0	4	4
	GI	Bulgaria > Romania > Germany > UK > France	Ireland > Italy > Netherlands > Spain > Poland > Denmark	0	4	6
Illegal	**NB**	Italy > UK > Germany	Spain > France		3	
	Q	Italy > UK > Germany	Spain > France > Greece	0	3	1
	GI	Italy > UK > Germany	Spain > France > Greece > Finland > Sweden	0	3	3
	MNB	-	UK > Germany > France > Italy	2	−3	−1
TAF	**NB**	-	Poland > Lithuania			
	Q	-	Poland > Finland > Lithuania	1	0	1
	GI	-	Poland > Finland > Lithuania	1	0	1
Wild boar	**NB**	Finland	Romania > Latvia > Poland			
	Q	Finland > Romania	Latvia > Poland	0	1	−1
	GI	Finland > Romania	Latvia	0	1	−2

A sensitivity index was also calculated to assess the magnitude of the influence of the categorization methods on each module results. This sensitivity index (SI) was computed after considering the number of countries for which the risk score changed "CC", the extent of this change "EC" (e.g., from 2 to 5, three levels of change), and the total chances of change for this pathway "TC". This TC represents the sum of the potential levels of change for all the countries by this pathway (e.g., a country with an initial risk score of 3 has a maximum of three possible levels of change, from 3 to 0, whereas a country with a risk score of 1 has up to four possible levels of change). The sum of the possibilities for all the countries composed the TC for this pathway. The SI was computed as follows: $SI = \frac{\sum LC * CC}{TC}$

Results

The model and its results are available as an Excel file in a public domain (www. asfrisk.eu) and as Additional file 1. The risk scores of ASFV introduction by the five pathways into each EU member state are presented in the results column on each pathway spreadsheet, and are also summarized in Table 2 and in the "overall results summary" worksheet of Additional file 1.

The list of countries at higher risk (risk scores equal to 4 or 5) per pathway is provided in Table 2 in descending order, from highest to lowest risk (the natural breaks row). These are the countries that would benefit from prevention activities for each analyzed pathway. The average number of countries with high-risk scores per pathway was 3.6, and there were some pathways with only two countries at higher risk (transport fomites), whereas others had five (legal pigs) or four (illegal and wild boar) countries at higher risk.

Different distributions of risk scores were observed between pathways (Figure 3). Whereas most countries presented a risk score of 0 (legal pathways) in some analyzed pathways, the majority of countries presented a risk score of 2 in others (illegal imports and transport fomites). Interestingly for these last two pathways, none of the countries presented the highest risk score of 5.

Figure 3 Distribution of risk scores per pathway. The number of countries per risk score was represented for the five assessed pathways.

Fifteen EU member states (55.5%) had at least one risk pathway with a risk score equal to or higher than 3(Figure 3). Thirteen countries (48%) presented one risk score of 4 or 5. Four countries had a risk score of 5 for one pathway: Bulgaria for legally imported products during the HRP; Finland for wild boar, Slovenia and Sweden for legally imported pigs during the HRP. However, these countries did not necessarily obtain high risk scores in other pathways; indeed some (Bulgaria, Slovenia and Sweden) obtained risk scores of 0 and 1 for other pathways.

Among the 12 countries with lower risk scores (all risk scores lower than 3), some countries presented similar risk profiles, with the same risk scores for the same pathways.

Sensitivity analysis

The results compiled in Table 3 reflect that for all the pathways and categorization methods, only two pathways presented differences in the order of countries at higher risk. In the TAF pathway, Lithuania and Finland interchanged their positions from the second to the third, and vice versa, when applying quantiles and geometric intervals instead of Jenks NB. For the illegal pathway, modifications in the order were observed when comparing Manual NB (the reference method) with the other categorization methods (Jenks NB, quantiles, geometric interval). Italy became the first country at risk (instead of forth by manual NB), and France came fifth (instead of third by manual NB) when using other methods. Consequently, the positions of the other countries changed, but not their relative order. For the other pathways, the only observed changes related to the inclusion of countries in category 5 (the countries considered at risk 4), or to those included in category 4.

The SIs calculated by the pathway and categorization method are presented in Table 4. The pathway that underwent the greatest modifications in terms of extent of change (EC), unlike the others, is that associated with legal import of products, which presented an average SI over all categorization methods evaluated at 34. This was also the only pathway for which some countries (8) underwent the maximum EC at three levels. In the remaining pathways, risk scores were less sensitive, with an SI of 8 (Legal imports of pigs and Transport pathways), 11 for the wild boar module and 12 for Illegal transport. The maximum EC observed in the other pathways was 2 levels, which affected four countries (legal imports of pigs) and one country in the illegal pathway. In the TAF and wild boar pathways, the countries' risk scores changed one level at the most.

Table 4 Sensitivity indices obtained in the sensitivity analysis of each pathway and categorization method

	SI Quantiles	SI Geometric Interval	SI Manual Natural Breaks	Average SI per pathway	Average EC	Maximum EC (CC)
L. PIGS	10.9	4.2	NA	7.6	1.2	2(4)
L.PRODS	31.4	35.5	NA	33.5	2	3(8)
Illegal	9.5	13.7	12.6	11.6	1.1	2(1)
TAF	5.6	10.1	NA	7.9	1.0	1(9)
WB	5.6	16.7	NA	11.1	1.1	1(6)

SI (Sensitivity Index), EC (Extent of change), CC (Number of countries that suffered this change); L.PIGS (Legal imports of pigs), L.PROD (Legal imports of products), ILLEGAL (Illegal imports of products), TAF (Transport associated fomites) and WB (wild boar); NA (Not applicable).

Discussion

The modular framework herein presented integrates the methodology and outputs from five pathway-specific risk assessments to produce specific risk profiles for ASFV routes of entry into the EU. All the modules used the best data available, which need to be consistent and homogenously collected across EU member states. Due to the limited availability of detailed data and the huge variability between EU member states in terms of pig production characteristics, trade, environmental and even socio-cultural features, the results generated by this modular risk assessment framework need to be interpreted with caution. Some of the EU databases used for model parameterization were not as complete and detailed as national ones [18]. It has to be stated that this may have resulted in under- or overestimations of risk scores

for some countries, depending on the reliability of the data available per country in these generic databases. Data on wild boar density and abundance of backyard pig production were limited at the time of the analysis performance, and could definitely affect the model results and the next assessment steps (backyard data could especially affect the future exposure assessment) [21]. The incorporation of recent FAO data on wild boars and low biosecurity farm [39] or wild boar density models based on ecological parameters [40] may improve the analysis. However, as the unit of analysis of this framework is the national level, these data are not expected to influence the final analysis outcome, especially when we consider that the densities of wild boars or domestic pigs in the individual sensitivity analysis of the wild boar pathway were the risk factor with the lowest influence on the model's results [22].

The present framework integrates the calculations and results of five risk analyses using diverse methodological approaches for risk estimation. Legal import pathways were estimated by using stochastic quantitative models; consequently, an absolute probability of the risk of ASFV entry was obtained per country (available in Additional file 1). Only for this particular case can we state that the risk of ASFV introduction through legal imports of products during the HRP was higher than by legal imports of pigs during the same period. The probability of ASFV introduction through legal imports of pigs during the HRP was extremely low (the maximum probability was 0.000393 estimated for Sweden which, on average, corresponded to one introduction every 2544 years), while for the legal imports of products during the HRP it was much higher, with a maximum probability of 0.226 for Bulgaria (one expected introduction 4.4 years on average). These probabilities referred only to the risk of ASFV being released into the EU by these routes, without considering the probability of exposure, which could certainly modify the final risk of ASF outbreak occurring in the country. However, as the main goal of this work was to develop a method to integrate different types of model (fitting different data types and qualities) into the same framework, the probabilistic results were transformed into risk scores from 0 to 5, and took the same format as the other pathways. Nevertheless, both the detailed risk probabilities and any additional information can easily be consulted in the modular framework provided (see Additional file 1).

For the other pathways, estimated semi-quantitatively, it was not possible to make comparisons between routes, but between countries within the same route. The methodology selected for the semi-quantitative assessments differed substantially from the conventional methods, which combined matrices of numbers and risk terms. Risk matrices can provide an impression of higher accuracy and/or confidence if compared with qualitative assessments, which can be particularly wrong when scores are assigned and combined arbitrarily, and with no transparency [41]. In contrast, the systematic approach used herein produced risk scores by the weighted linear combination of the selected parameters contributing to the risk. The structure of these calculations, data inputs and weights used in the framework are presented in Additional file 1, thus ensuring the transparency of the model methods and results.

Nonetheless, as with any risk assessment model, a certain level of subjectivity was involved in selecting the pathways analyzed (based on the literature review on the ASFV transmission mechanisms, routes of introduction into previously free areas and the current epidemiological situation), module structure, methods applied, the parameters used as risk indicators (based on data availability), and categorization and weighting methods (analyzed in the sensitivity analysis). Attempts were made to minimize these limitations by means of the model's transparency and the systematic application of the chosen approaches, which occurred with data categorization. After testing several categorization methods, natural breaks were used as

this is the method that best adapts to the different distributions presented for all the input data. Within this method, the optimization method using Jenks algorithm was employed for most pathways to minimize intra-class variance and to maximize inter-class variances [24]. For the illegal import pathway however, the very skewed distribution of some parameter values led to relatively high-risk scores when Jenks adjustment was utilized, and it was thus decided to use manual natural breaks for this module. This observation is confirmed when comparing the list of countries at higher risk by each categorization method within illegal pathway (Table 2). The use of Manual NB provides a maximum risk score of 4 for four countries, whereas with Jenks NB three countries presented a risk score of 5. The lists of countries at higher risk observed among the two NB categorization methods were very similar, with two countries that modify their order and consequently change the position of the others but not their relative risk. Also the magnitude of the changes was not high (SI: 12.6) when comparing with other pathways. Consequently, although the magnitude of changes observed was not big, authors decided to employ Manual NB for this pathway for calculating the final results, as it better fits to the distribution of input data used and provides more adjusted results.

The sensitivity analysis assessed the impact of the categorization methods on the model's outcomes and suggested satisfactory model robustness for all the pathways, except for the legal imports of products, which presented the greatest EC, with an average SI of 34. In addition, a maximum extent of change of 3 levels took place for eight countries for the legal imports of products pathway when modifying categorization. Within this pathway, data categorization was used only for transforming risk probabilities into risk scores, but conferred no additional value to the calculations (see the model structure in Figure 1). Consequently for this specific case, evaluating the absolute probabilistic values presented in Additional file 1 is recommended to better interpret the results instead of risk scores.

For the remaining pathways, the variations found by using other categorization methods were much slighter (SI less than 12), with a maximum extent of 2 levels of risk (legal imports of pigs during the HRP and illegal imports) and 1 (transport fomites and wild boar). This confirms the robustness of the model structure, especially for the semi-quantitative pathways where data categorization proved an important element to assess risk.

Besides the analysis of the EC observed (SI), the comparison of the main study output, specifically the list of countries at highest risk per pathway, revealed good consistency among the different categorization methods employed. Indeed the order of countries at highest risk changed only for the illegal pathway (when using manual NB, as previously discussed) and for TAF (when quantiles or geometric intervals were employed). Specifically for TAF, the values of two countries interchanged from second and third positions at risk, but both remained in the same risk category. For all the other pathways, no changes were observed in the list of the highest risk countries, except for the inclusion of some countries at the end of the list. These results suggest that the main model's outputs were not influenced when other categorizing methods were used.

All five modules within the framework provided relative risk scores on a scale of risk scores from 0 to 5, the equivalent to risk probabilities from negligible to very high. Despite the use of different methodologies for each pathway module inhibits a direct comparison of the results among the pathways, as the same methodology was used for all the countries within each module, it was possible to compare the countries' risk scores for a given pathway. Accordingly, these results can be used at the EU level to determine the countries/areas at

higher risk per route of introduction. The application of specific control measures (adapted to the pathway's origin of this risk) in these high-risk countries/pathways could prove beneficial for the whole EU. Based on this assumption, special attention should be paid to Sweden and Slovenia (risk of 5), followed by Finland, France and Greece (4), for the legal import of pigs pathway during the HRP. In relation with legal imports of products during the HRP, the only country at high risk was Bulgaria (5), followed by Romania and Germany (4). For illegal imports, no country presented the highest score (5), but a risk score of 4 was estimated for France, Germany, Italy and the United Kingdom. Something similar occurred with the transport associated fomites pathway, for which Lithuania and Poland obtained the highest scores (4). Finally, the risk deriving from the wild boar movement route was estimated with a score of 5 for Finland, followed by Latvia, Poland and Romania with a risk score of 4.

Some countries, like Finland, Romania, Germany, Poland and France, obtained risk scores of 4 and/or 5 for several pathways. These countries would benefit from further national research to elucidate which pathways are at higher risk for their country and which actions can be implemented to prevent these risks. Countries with similar risk profiles are also often similar in terms of external trade, geographic location, wild pig movements, and other factors that have been considered in the assessment. Germany and the UK obtained high risk scores for illegal imports, legal import and transport, which reflect their vast volume of trade with third countries [27]. They operate more flights from ASF-affected countries, and they also obtained the largest number of residents from ASF-affected countries, and very high values for inbound and outbound tourism (EUROSTAT data). In comparison to other EU countries, these two countries maintain very close relations with non EU countries, which could potentially incur a risk for disease introduction.

Transport and wild boar obtained risk scores of 4 or 5 in Lithuania, Finland or Poland, which reflects their geographical proximity to affected areas and, consequently, the facility of entrance by these routes influenced by distance. These results have been recently validated by the notification of ASF cases in dead wild boar in territories of Lithuania and Poland [9]. Malta and Luxembourg, with almost no pigs and an insignificant number of imports and risk relations, were at extremely low risk (around 0) for all the analyzed pathways.

In addition to these logical and biological arguments, the results obtained in the present framework agree with previous country-specific risk assessments. In a previous risk assessment for Finland, introduction by wild boar was considered to be the riskiest pathway [13], which agrees with the risk score of 5 obtained in the wild boar module of the present framework. Similarly, Germany obtained a risk score of 3 in the present transport fomites module and a moderate-high risk for the risk of the means of transport of pigs in the national assessment made by FLI [14]. These examples stress that despite the framework's limitations, the obtained results agree with other assessments. This, consequently, inspires confidence in our model's results. However, the authors are aware that these agreements do not guarantee the exact prediction of risk.

The analysis of the present results per pathway also provides interesting information on risk management. In some of the pathways analyzed, risk concentrated mainly in very few countries (transport fomites in two countries), whereas other pathways proved relevant for many countries (legal imports of pigs, illegal imports and wild boar). These findings suggest the benefits of a coordinated EU program for preventing and controlling the disease.

The outcomes obtained with this modular framework can be used to inform about the development of targeted risk management measures in the EU by implementing preventive measures in those pathways and countries that obtained higher risk scores. Additionally, the modular framework provided in the additional file can be used as a template to estimate the risk of ASFV introduction into other geographic areas or timeframes as more data become available.

Conclusions

A modular framework has been implemented to estimate the risk of ASFV entry into the EU through five different introduction routes: the legal imports of pigs and products during the high risk period, the illegal imports of products, the transport associated fomites and the movement of wild boar. The framework, available in a public domain, integrates the five risk assessment modules and offers a transparent, easy-to-interpret tool that can be easily updated as data become available and can also be adapted to other regions of interest. The model's results identify the EU countries at higher risk per route of ASFV introduction, and acts as a useful basis to develop a coordinated EU program and to, ultimately, improve ASFV prevention in the EU.

Endnotes

[a]Croatia was the EU country not included in the analysis given its recent inclusion in the EU (1/7/2013).

Competing interest

The authors declare that they have no competing interests.

Authors' contribution

This work is the result of the long-term coordinated efforts made between three institutions (RVC, CISA-INIA and UCM). LM, BML, MM and JMSV carried out the analysis for the legal imports and transport fomites pathways. SC, BJ, FSV, DUP and BW made the illegal assessment. AT, MM and MJR developed the assessment for the wild boar pathway. The respective teams did the sensitivity analyses in the corresponding pathways (varying the categorization methods) and adapted their pathway analysis in order to integrate a common structure. LM integrated the modules into the common structure and wrote the draft of this manuscript. All the authors participated in the discussions about the selection of the methodologies used, the interpretation of the results, and the preparation of the manuscript by reviewing the results, and by notably modifying and suggesting changes to improve the study. All authors read and approved the final manuscript.

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Additional file

Additional_file_1 as XLS

Additional file 1 Modular framework for estimating the risk of ASF introduction into European Union. The present file includes input data, calculations and results of the five pathways assessed in the study. In addition, explanations about the structure and source of data were also included in separate sheets.

Figure 1: Detailed structure of the modular framework. The five risk pathway modules are represented and include the main steps of the respective quantitative and semi-quantitative models. P (Probability in the quantitative assessments, Proxy in the semi-quantitative assessments), RV (Risk Value), JENKS NB (Jenks Natural Breaks), WLC (Weighted linear combination of values), Manual NB (Manual Natural Breaks), X (Multiplication of values) + (sum of values).

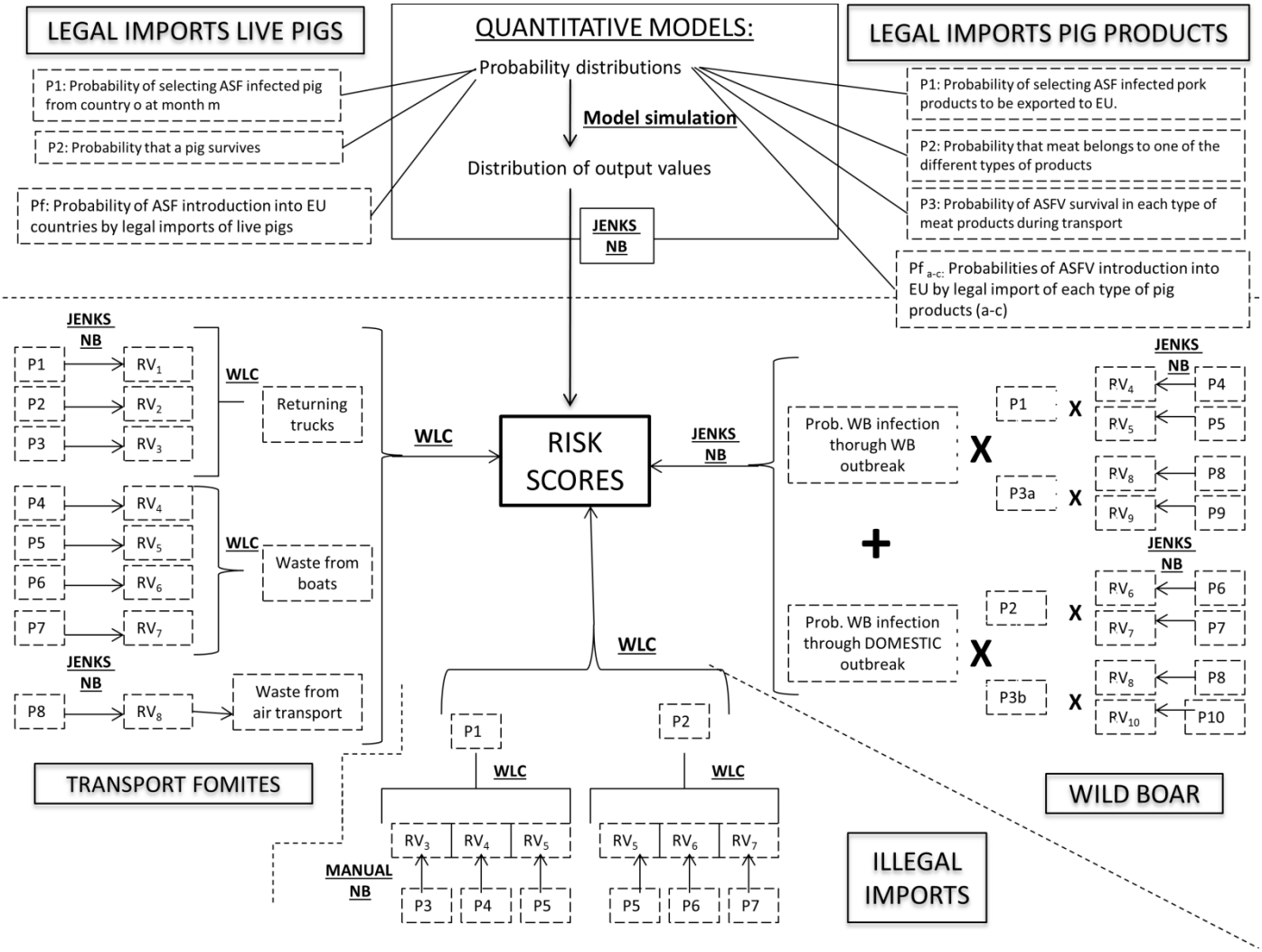


Figure 2: Scenario tree for the legal import of pigs and legal imports of products pathways.

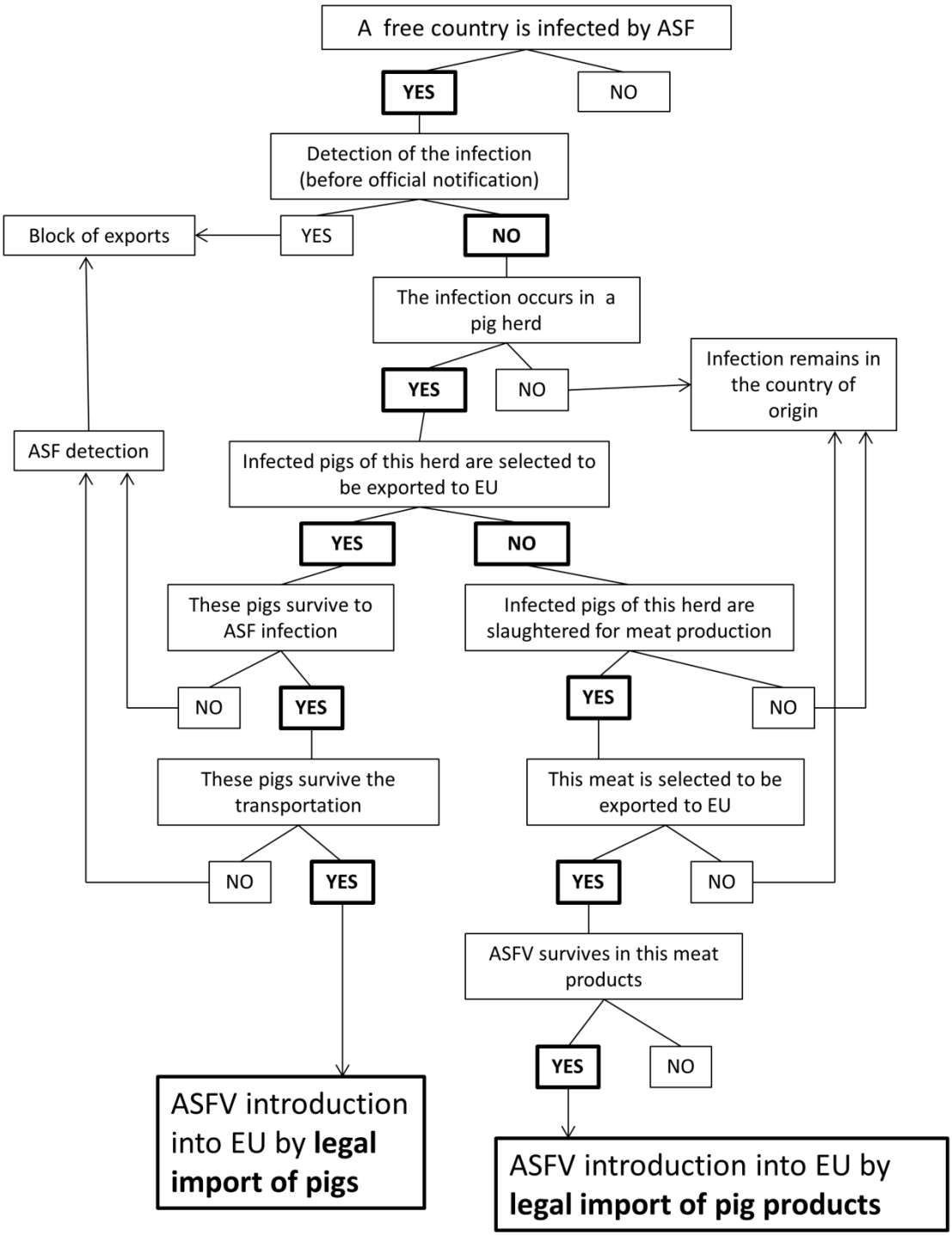
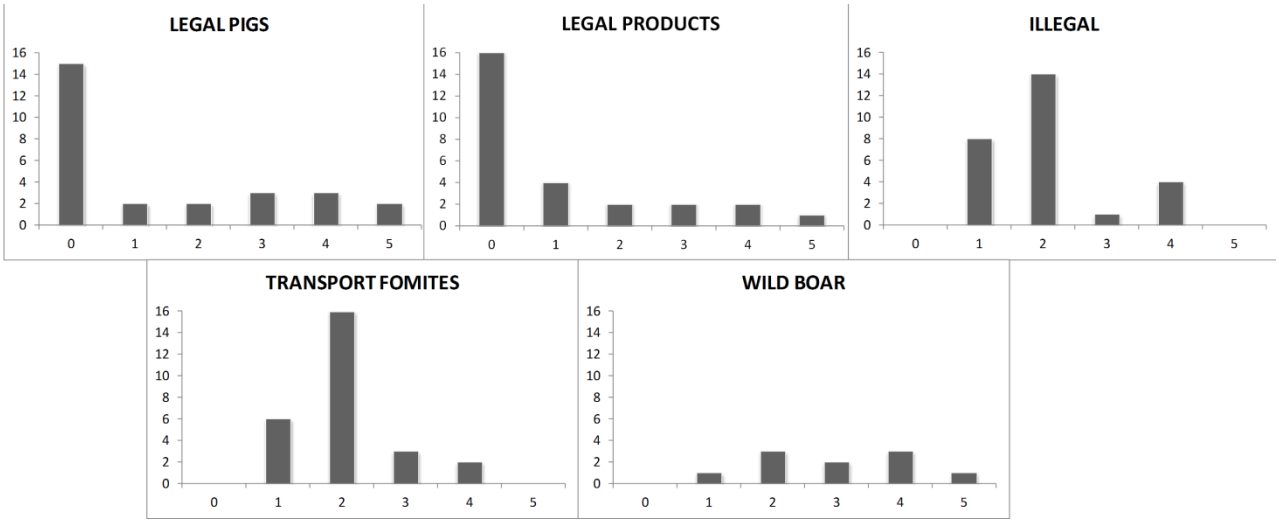


Figure 3: Distribution of risk scores per pathway. The number of countries per risk score was represented for the five assessed pathways.



OBJETIVO 3: Desarrollo de modelos para la evaluación de factores de riesgo para el control de la PPA en zonas endémicas

La PPA es una enfermedad que presenta una epidemiología compleja, con la intervención de hospedadores domésticos, silvestres, vectores y una importante relevancia socio-cultural. Todos estos factores deben ser estudiados y considerarse en el diseño e implementación de los programas de control y aún más en zonas endémicas, ya que su presencia dificulta en gran medida la erradicación de la enfermedad.

Este objetivo incluye la identificación y evaluación de factores de riesgo presentes en una zona endémica para PPA (Cerdeña) mediante el desarrollo y combinación de modelos matemáticos (3.1). A su vez, se realizó un estudio retrospectivo con el fin de valorar el papel del jabalí en el mantenimiento de la PPA en un territorio (3.2).

Objetivo 3.1

Para ello, se evaluó la evolución histórica de la PPA en Cerdeña, donde a pesar de los grandes esfuerzos y fondos invertidos para luchar contra la enfermedad, la PPA continua estando presente desde 1978. Se realizaron estudios espacio-temporales de los brotes de la enfermedad, analizando las características del sector porcino, los planes de erradicación y medidas de control implementadas. En base a ello, se identificaron los siguientes factores de riesgo para la difusión y mantenimiento de la PPA en Cerdeña: i) el bajo nivel de profesionalización de la producción porcina, con granjas de muy pequeño tamaño, con escasa o nula bioseguridad, donde el cerdo es una fuente de ingresos secundaria y el comercio ilegal de cerdos y sus productos es frecuente; ii) la presencia de cerdos pastando en libertad en territorios comunales. A pesar de estar prohibido a día de hoy (los denominaremos cerdos ilegales), su presencia continua en la isla; y en último lugar, iii) los jabalíes, abundantemente presentes en Cerdeña, afectados por la PPA, cuyo papel aún no está del todo elucidado.

Con el objetivo de evaluar esta hipótesis, se desarrolló un estudio que combinaba la aplicación de un modelo de difusión de PPA en los cerdos domésticos, con modelos de regresión logística para evaluar la influencia de los factores de riesgo considerados más importantes para la ocurrencia de la enfermedad. Se simuló la difusión de PPA mediante un modelo estocástico espacial empleando los datos de las granjas y movimientos del año 2012. Este modelo señaló un bajo impacto de los brotes de PPA (1,7 granjas infectadas de media por brote), ocasionados principalmente por la difusión local del vPPA (99% de los brotes ocasionados por esta vía). Estos

resultados fueron contrastados con la magnitud de las epidemias reales de PPA revelando gran similitud entre ambas y destacando la importancia del sector doméstico y la bioseguridad en granja en la difusión habitual de la enfermedad. En paralelo, se evaluó la influencia de los cerdos ilegales y la densidad de jabalíes en la ocurrencia (aparición) de los brotes de PPA mediante el desarrollo de cuatro modelos de regresión logística. En todos ellos, ambos factores resultaron significativos para la ocurrencia de PPA, teniendo la presencia de ilegales más peso, excepto en los casos de muy alta densidad de jabalíes, en las que esta influía de gran manera.

Estos resultados, fueron empleados en un modelo para evaluar su influencia en la aparición y difusión de los brotes de PPA para la realización de mapa de riesgo de difusión y ocurrencia de PPA en Cerdeña, donde se observan las zonas más afectadas para cada factor. Estos resultados demuestran la importancia en Cerdeña de implementar unas medidas de bioseguridad estrictas en granja que limiten la difusión de la enfermedad, y la necesidad de aplicar programas para controlar tanto los cerdos ilegales como el potencial contacto del jabalí al doméstico especialmente en zonas de alta densidad.

Objetivo 3.2

Dada la importancia y papel de la vida silvestre en el mantenimiento y difusión de enfermedades, se llevó a cabo un estudio con carácter retroactivo para evaluar el papel del jabalí en la persistencia del vPPA en el ambiente tras la erradicación en el cerdo doméstico. Para ello, se analizaron 158 muestras de jabalíes procedentes del Parque Nacional de Doñana, una de las últimas regiones de España donde la PPA fue erradicada, donde había presencia de garrapatas, jabalíes y producción de cerdo en extensivo. Se realizaron análisis serológicos y virológicos en las muestras obtenidas entre 2006 y 2010 para la presencia de PPA, según las técnicas recomendadas por la OIE. Todas las muestras analizadas resultaron negativas tanto a detección de ácidos nucleicos como a anticuerpos frente a PPA.

Estos resultados confirman la ausencia de infección en jabalíes de una de las áreas en su día más afectadas por la PPA en España, sugiriendo que los jabalíes juegan un papel poco importante en la persistencia del vPPA en el ambiente. Estos resultados concuerdan con los estudios realizados por otros autores sugiriendo que el vPPA no persiste en poblaciones aisladas de jabalíes por largos periodos de tiempo sin la presencia de otros factores como la reinfección por contacto con cerdo doméstico o productos contaminados.

Los resultados obtenidos en estos estudios proporcionan un conocimiento fundamental sobre el papel de los distintos factores de riesgo en la epidemiología de la enfermedad en escenarios concretos. Estos resultados, así como las metodologías aquí propuestas podrán ser aplicados en otras regiones endémicas para PPA para evaluar sus particulares factores de riesgo. La posterior adaptación de los programas de control en función de los resultados obtenidos, permitirá priorizar las medidas de control y erradicación en las zonas/ factores señalados, con el fin último de lograr un control de la enfermedad en la zona.

***Artículos científicos incluidos en la tesis*:**

- Mur L., Atzeni, M., Martínez-López, B., Feliziani, F., Rolesu, S., Sánchez-Vizcaíno, J.M., 2014. **35-year presence of African swine fever in Sardinia: history, evolution and risk factors for disease maintenance**. *Transb Emerg Dis*. Aceptado para su publiuación.
- Mur L., Fernández-Carrión E., Atzeni M., Rolesu S., Feliziani F., Sánchez-Vizcaíno JM., Martínez-López B., 2014. **Combination of simulation and statistical modeling to identify factors contributing to ASF endemicity in Sardinia**. En preparación.
- Mur L., Martínez-López B., Gallardo C., Gortazar C. and Sánchez-Vizcaíno JM., 2012. **Monitoring of African Swine Fever in the Wild Boar Population of the Most Recent Endemic Area of Spain**. *Transb Emerg Dis*. 59, 526–531.

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5. Publicaciones

Transboundary and Emerging Diseases

REVIEW ARTICLE

Thirty-Five-Year Presence of African Swine Fever in Sardinia: History, Evolution and Risk Factors for Disease Maintenance

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Summary

Despite the implementation of control efforts and funds to fight against the disease, African swine fever (ASF) has been present in Sardinia since 1978. It has caused serious problems for both the industrial pig sector and the regional authorities in Sardinia, as well as the economy of Italy and the European Union, which annually supports the costly eradication programme. During this time, ASF has persisted, especially in the central-east part of Sardinia where almost 75% of the total outbreaks are concentrated. The Sardinian pig sector is clearly divided into two categories based on the specialization and industrialization of production: industrial farms, which represents only 1.8% of the farms in the island and non-professional holdings, which are comprised of small producers (90% of pig holdings have <15 pigs) and apply little to no biosecurity measures. Additionally, illegally raised pigs are still bred in free-ranging systems in certain isolated parts of the island, despite strict regulations. The illegal raising of pigs, along with other high-risk management practices (e.g. use of communal areas) are likely the primary reasons for endemic persistence of the virus in this area. The compensation provided to the farmers, and other aspects of the eradication programme have also negatively influenced eradication efforts, indicating that socio-cultural and economic factors play an important role in the epidemiology of ASF on the island. The aim of this study was to comprehensively review the evolution of the 35-year presence of ASF in Sardinia, including control measures, and the environmental and socio-economic factors that may have contributed to disease endemicity on the island. The present review highlights the need for a coordinated programme that considers these socio-economic and environmental factors and includes an assessment of new cost-effective control strategies and diagnostic tools for effectively controlling ASF in Sardinia.

Introduction

African swine fever (ASF) is a viral disease that affects swine. It was first discovered in Kenya in 1921, and thereafter, the African swine fever virus (ASFV) spread throughout the African continent. It was subsequently introduced into Portugal in 1957 but was rapidly controlled; in 1960, it was re-introduced into Portugal and then spread to Spain. During the 1970s and 1980s, ASF was widely present in the Iberian Peninsula (i.e. Spain and Portugal) and affected a large number of pig holdings with tremendous economic consequences for both countries. At that time, while the disease was endemic in the Iberian Peninsula, many other countries were affected by sporadic outbreaks. Most of

these outbreaks were caused by the introduction and use of food waste from international planes or boats to feed pigs. Some examples of these terrible outbreaks include Brazil, Cuba and the Dominican Republic, while other European outbreaks were quickly controlled in France, Belgium, the Netherlands and northern Italy in 1967 and 1969 (Costard et al., 2009). Sardinia was firstly affected in March 1978, most likely by contaminated waste fed to pigs either in the port of Cagliari or the military airport of Decimomannu (in the province of Cagliari) (Anon., 1978). Today, from among these, many affected territories of the 1960s, 1970s and 1980s, Sardinia is the only region where the disease remains endemic.

Since the early introduction of ASFV in Sardinia, disease presentation was similar to what was observed in Spain and Portugal. Specifically, ASF was present in its acute form: characterized by the presence of erythema, cyanosis and extensive haemorrhages, mainly in lymph nodes and spleen. However, as in Iberian Peninsula, not all infected pigs die from the disease. Some pigs suffer from the disease, but survive infection, thus entering a carrier state, which certainly plays an important role in the spread of this disease (Wilkinson, 1984).

Molecular studies have revealed that the ASFV isolate present in Sardinia belongs to Genotype I (vp72), the same genotype present in Spain and Portugal, which also escaped to South America and other European countries (Bastos et al., 2003). Additional studies have focused on other regions of the genome (p54 gene), and they have classified Sardinian isolates within Genotype Ia. These isolates are very similar to both European and American isolates, but they are quite different from West African ones (Gallardo et al., 2009).

African swine fever affects both domestic pigs and wild boars in Sardinia. The wild boar population in Sardinia is abundant (>70 000 animals; Firinu and Scarano, 1988) because favourable habitat is extensively available on the island. Early European studies demonstrated that wild boars are susceptible to ASFV infection, with high mortality observed in the infected wild populations (Polo Jover and Sánchez-Botija, 1961; Ordas et al., 1981). In Sardinia, the mortality was much lower, with antibodies detected in approximately 5% of apparently healthy wild boars. This supported the hypothesis that wild boars become free of the virus in a short time (Firinu and Scarano, 1988). As a result, further studies expanded on the secondary role that wild boars play in the epidemiology of the disease in Sardinia (Laddomada et al., 1994; Rolesu et al., 2007), and Spain (Perez et al., 1998; Mur et al., 2012). In Sardinia, it has been proposed that wild boars usually become infected by direct contact with infected domestic pigs or by indirect transmission from infected carcasses, offal, etc., and that they are unable to maintain the disease if no continuous viral source is present (e.g. infected domestic pigs coming into contact, infected carcasses, offals, etc.).

It has been well documented that the soft tick of the *Ornithodoros* genus (*O. erraticus*) plays an important role in ASF epidemiology in many regions, including the Iberian Peninsula (Oleaga-Perez et al., 1990; Arias and Sánchez-Vizcaíno, 2002; Sánchez-Vizcaíno and Arias, 2012). For that reason, since the Sardinia control programme of 1982 (Regione Autonoma de la Sardegna [RAS], 1982), researchers looked for the presence of ticks on the island that are capable of maintaining the disease. In particular, field research to find *Ornithodoros* ticks was performed in 357 holdings from 20 different districts in the Province of Nuoro in the 1980s, showing negative results (Ruiu et al., 1989). Thus, control of soft ticks has not been considered for the ASF mitigation on the island, although further confirmation using currently available techniques is highly recommended.

African swine fever has been present in Sardinia for more than 35 years, and although different control strategies have been adopted, some risk factors (socio-cultural, environmental, etc.) remain present on the island and these impede successful eradication plans (Martínez-López et al., submitted). This work reviews the evolution of ASF in Sardinia by addressing all the factors considered important for disease maintenance and focuses on specific characteristics of the Sardinian pig sector, as well as the control measures applied to date. In addition, it provides some information about new mitigation techniques available and specific recommendations.

Swine Production in Sardinia

Swine husbandry has been present in Sardinia since the sixth century BC when it was introduced by the Neolithic people (Albarella et al., 2006). However, the pig sector has always been a secondary activity when compared with other more important livestock production systems on the island, such as sheep and goats (RAS, 2004). The size of the swine sector has slightly decreased in recent decades since the initial peak in 1980 (ISTAT, 2013). However, the particulars of the sector have been maintained, such as widespread piglet (5–8 kg) production for human consumption, the use of free-ranging pigs in communal territories, and other strategies that will be discussed in more detail later.

In 2012, the Sardinian census reported approximately 200 000 pigs located in roughly 15 000 holdings (national database, NDb). More than 88% of these holdings (n = 13 650) had <15 adult pigs (Fig. 1), and 51% had ≤4 pigs (NDb, 2013). The average number of pigs per holding was 13.5 pigs, but large differences were found among different provinces (range: 6.9–63.9). However, it is important to note that the number of pigs included in the census does

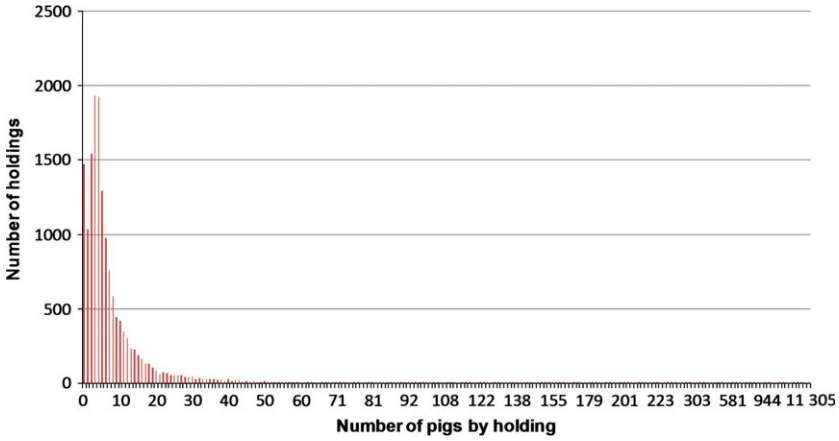

Fig. 1. Number of pig holdings per number of pigs presented in it.

not accurately represent the true population because a very large number of pigs are illegally bred without being identified or registered.

Pig holdings are not equally distributed over the Sardinian territory (Fig. 2). The median number of pig holdings per district is 41.5 (range: 1–357), with the highest density of holdings located in the northern region. 49% of pigs are bred in the provinces of Cagliari and Medio Campidano, where farms are larger; yet Oristano and Sassari have the largest number of holdings. Indeed, most industrial pig

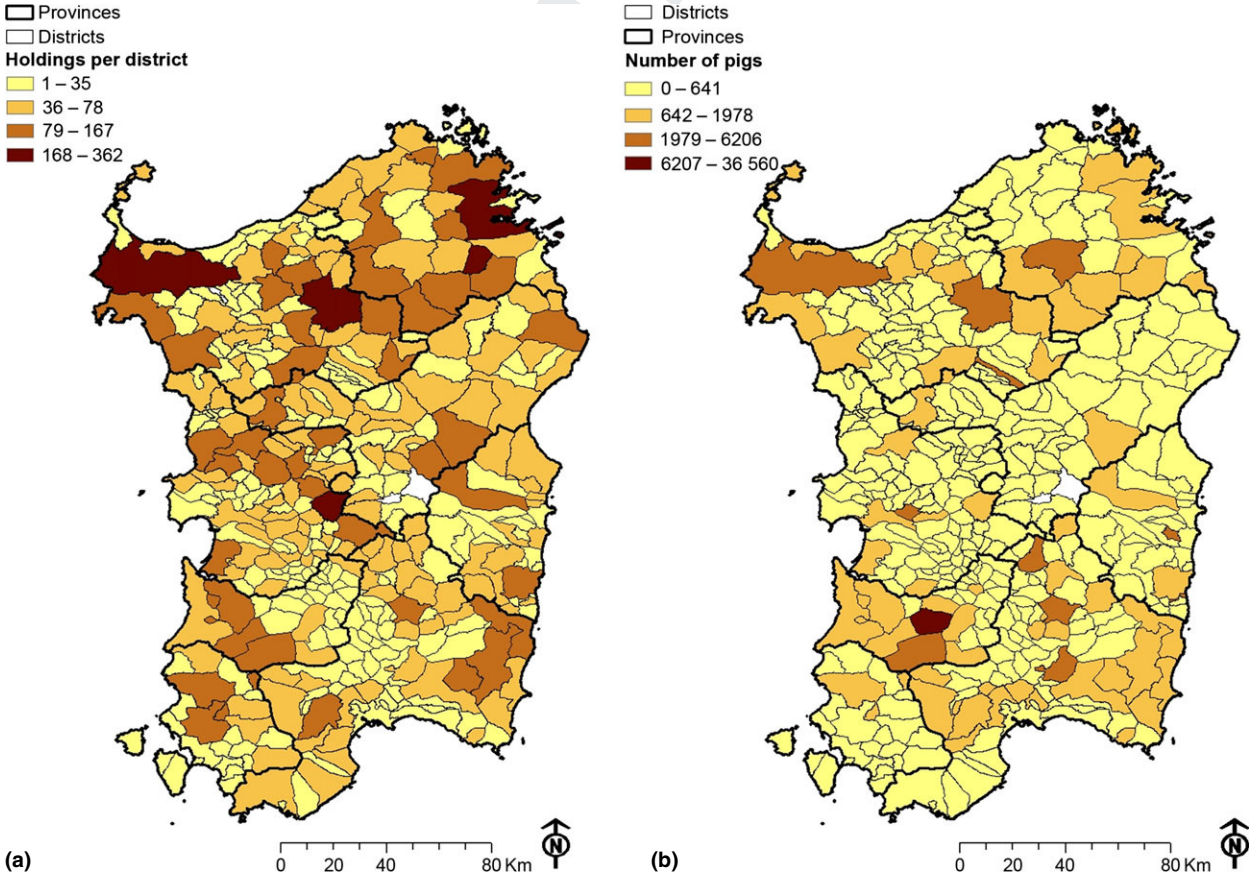

Fig. 2. Density of pig holdings per district (a) and density of pigs per district (b).

Colour online, B&W in print

farms, with more than 500 pigs, are located on plains of the island.

The remaining pig production (not considered industrial given the small number of pigs and the poor biosecurity measures) is found in different types of small-scale farms. Family production is the most widely distributed over the whole territory. This type of holding, with typically <4 adult pigs, is not permitted to move animals because the ultimate aim is subsistence farming (consumption of piglets or products produced). Small-scale farms for reproduction or fattening also exist, with pigs kept confined within a structure or in semi-free-range situations, where pigs are kept within fenced land.

Additionally, a large number of pigs are bred illegally in free-range systems controlled by the farmer. This illegal practice is very common, especially in the central-eastern part of the island, but can also be found in other isolated, mountainous regions. These free-range herds can range from 2 to 300 pigs, and they eat the natural resources present in scrub lands and non-cultivated territories, such as acorns from evergreen oaks or chestnuts. These territories are often communal lands used traditionally and freely by neighbours for rearing their livestock. This practice is a source of income in economically depressed areas where very few species of animals (e.g. goats and cattle) can be bred due to the mountainous terrain. From the very beginning, this activity was considered controversial and has been frequently associated with the presence of ASF on the island (Mannelli et al., 1997, 1998).

For most pig producers in Sardinia (except for the industrial farms), pig production is a secondary activity and, approximately, 70% of these farms raise, more than one animal species on the same land. This is especially important in pasture areas with an altitude of ≥500 m, where pigs usually live together with sheep, goats or bovines (Porcu et al., 2004). This co-mingling of species and the high number of illegally produced pigs poses a major health risk not only for ASF, but also for other diseases, including some zoonoses such as *Trichinella* (Pozio et al., 2006).

The swine market in Sardinia, especially the industrial one, has been seriously affected by the presence of ASF on the island and resulting ban on swine exports (Italian Ministry of Health, Ordinance 19/03/1979). Nevertheless, a special derogation was established in 2005 (European Commission [EC], 2005a) that allowed the export of pig products obtained from Sardinian pigs bred outside the high-risk area, which involves specific biosecurity measures. This derogation ended in December 2011 whereby the EC expanded the high-risk area to include the entire territory of Sardinia, consequently forbidding any swine exports from pigs bred in Sardinia (European Commission, 2011). Currently, the only permitted swine exports include those products from pigs arriving from other territories of Italy. For example, in 2012, 62 500 pigs arrived from other Italian regions, like Perugia or Mantua, to be slaughtered and processed in Sardinia. These pigs could legally be exported from the island if they are processed and transported according to strict regulations that ensured the complete differentiation of this meat from that obtained from Sardinian pigs.

This complicated, controversial situation resulted in an inability to successfully compete with foreign markets and resulted in the closure of some of the largest pig production facilities in Sardinia in recent times (2011–2013). However, small local pig production is much less affected by these control measures. It is likely that the traditions and cultural factors associated with pig production and consumption (especially piglets, ham and other typical products obtained from free-range pigs), have allowed this local market to survive.

Historical Evolution of ASF in Sardinia

Temporal evolution

The presence of ASF on the island has not followed a continuous and predictable trend, presenting periods either of hyper- or hypo-endemicity, as seen in the epidemic curve (Fig. 3), likely strongly associated with sociologic and economic fluctuations on the island. From the very beginning (March 1978), ASF affected the southern region, mainly the province of Cagliari and caused the culling and destruction of >10 000 pigs. The disease in this area seemed easily confined, but in June 1978, ASF arrived in the province of Nuoro (Baggiani, 1978) where it seems to have found a favourable territory in which to persist. The number of outbreaks reported from 1978 to 1989 was not high, presumably due to a lack of understanding of the disease, the likely large number of unreported deaths in free-range pigs, and the absence of a real control program. In 1982, the first coordinated programme for controlling ASF in Sardinia was approved with funds from both the European Economic Community (EEC) and the national government (RAS, Legge 5/02/1982 n.6). This 5-year programme aimed to cull seropositive animals, improve diagnostic infrastructures (new laboratories) and implement strict control measures to fight against the disease (e.g.: 6-month period before repopulation of affected holdings, forbidding free-range pigs, etc.). Unfortunately, other important aspects were not included in this first plan, such as outreach and educational campaigns for farmers, or careful implementation of movement control measures. Farmers rejected these strict regulations and did not understand the reasons for culling apparently 'healthy' animals. In addition, the compensations for culled animals were frequently delayed, which further promoted resentment among farmers. As a result, some farmers hid their infected animals instead of

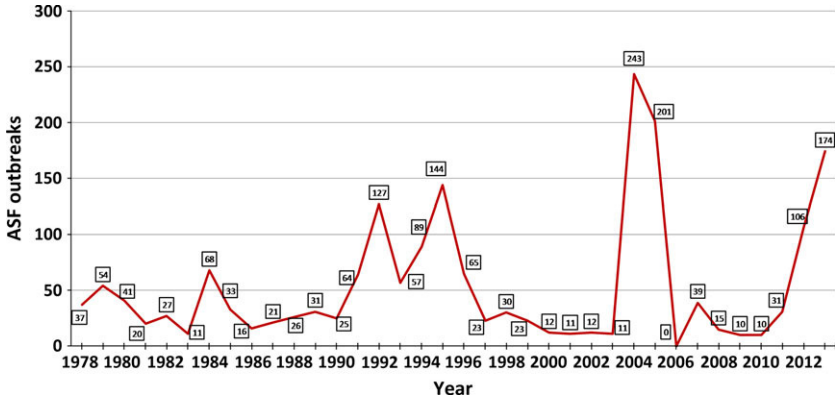

Fig. 3. Evolution of the number of African swine fever outbreaks per year in Sardinia.

declaring them, creating a perfect environment for the persistence and spread of the virus (Laddomada and Patta, 1988).

In the 1990s, the number of reported ASF outbreaks sharply increased and peaked with >140 outbreaks in 1995. Interestingly in 1990, the EEC approved new funds for the ASF eradication program in Sardinia (European Economic Community, 1990), which promoted the rapid culling of all potentially infected animals the extensive use of serology controls and the prompt compensation of culled animals. During this period, compensations to farmers increased and were accelerated in an attempt to encourage farmers to report the disease (Patta et al., 1994). These changes may have influenced farmers' behaviour and resulted in an increased number of diagnosed outbreaks.

After these years of hyperendemicity, the outbreaks appeared to be somewhat under control in the late 1990s and the very beginning of the twenty-first century, with approximately 10 outbreaks reported per year. The emergence of bluetongue virus in August 2000 dominated the attention of health authorities and ovine farmers for many years. However, during these years, the eradication of ASF in Sardinia seemed achievable, with a relatively stable hypo-endemic situation.

Unfortunately, the epidemiological scenario suddenly changed in 2004, when 243 outbreaks were declared, followed by 201 outbreaks the next year. Of all the outbreaks declared in 2005, 71% (n = 143) were reported from the province of Oristano, which had been relatively unaffected by the disease previously. In 2005, the EC approved new protection measures against ASF in Sardinia (European Commission, 2005b); consequently, the Sardinian region presented a new plan to fight against the disease, including more strict requirements for compensation after culling infected animals (Italian Ministry of Health, Ordinance 23/01/2006). These stringent changes in legislation may have influenced the decline in reported ASF outbreaks in 2006.

Since then, the situation seemed to remain hypo-endemic until the second half of 2011 when the number of outbreaks slowly increased and affected new parts of the island outside of the traditional endemic region. The EC responded by expanding the high-risk area for ASF to include the entire territory of Sardinia (European Commission, 2011). Accordingly in 2012, new control programmes were approved in June (RAS, 2012a) and December (RAS, 2012b). These new programmes significantly increased active surveillance activities in domestic and wild boar populations and implemented more restrictive requirements for pig transportation requirements, for the revocation of protected and surveillance zones, and also for the repopulation of pig holdings following disease outbreaks. Despite instituting these reforms, ASF outbreaks continued to occur in 2012 and 2013, occasionally in naïve areas such as the northern regions. Although some time is required to evaluate the effectiveness of this new control strategy, the role of the illegal free-range pigs or the presence of wild boars do not seem sufficient to explain the most recent evolution of ASF.

Spatial evolution

Unlike the temporal distribution, the spatial distribution of the disease has remained relatively stable over the past 35 years, affecting mainly the central-east (provinces Nuoro and Ogliastra and the bordering area of Sassari and Olbia-Tempo). Indeed, the data compiled since 1978 have revealed that 68.9% of outbreaks have been reported in the ancient province of Nuoro that included the current provinces of Nuoro, Ogliastra and a small part of Cagliari (Fig. 4).

During 1987, a hot spot in Nuoro and Ogliastra provinces was detected, which includes a series of districts with common borders and habitats (Fig. 5). This area is the traditional endemic area, where the disease has been

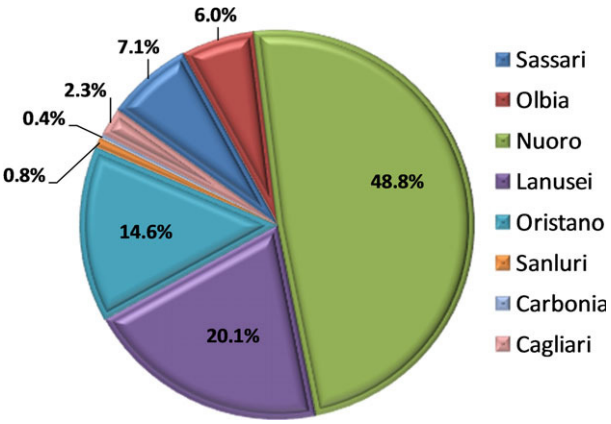

Fig. 4. Percentage of African swine fever outbreaks occurred per province in Sardinia from 1978 to 2012.

present for many years (e.g. ASF has been reported for 17 years in Villagrande). This suggests that the ASF problem may be particularly associated with specific practices or socio-economic factors in these areas. As previously mentioned (Fig. 2), this area does not correspond with the highest pig density, rather, it is hypothesized that certain practices and traditions have allowed the disease to persist (Martínez-López et al., unpublished observations).

We conducted a cluster analysis to identify the areas with significant space-time aggregation of outbreaks from 2004 to 2013. The space–time permutation model of SatScan v9.1.1 was used, with a temporal window of 3 months and no limited space window, using the database of ASF outbreaks. Based on this analysis, four statistically significant clusters were identified during the 2004–2013 period (Table 1).

Of the four clusters identified, only one was located in the traditional high-risk area for ASF in Sardinia (Cluster 2 in 2004). During the epidemic period of 2005, apart from the endemic province of Nuoro, some areas in the Oristano province were widely affected, representing the primary aggregation of the disease (Fig. 6). The fact that three of

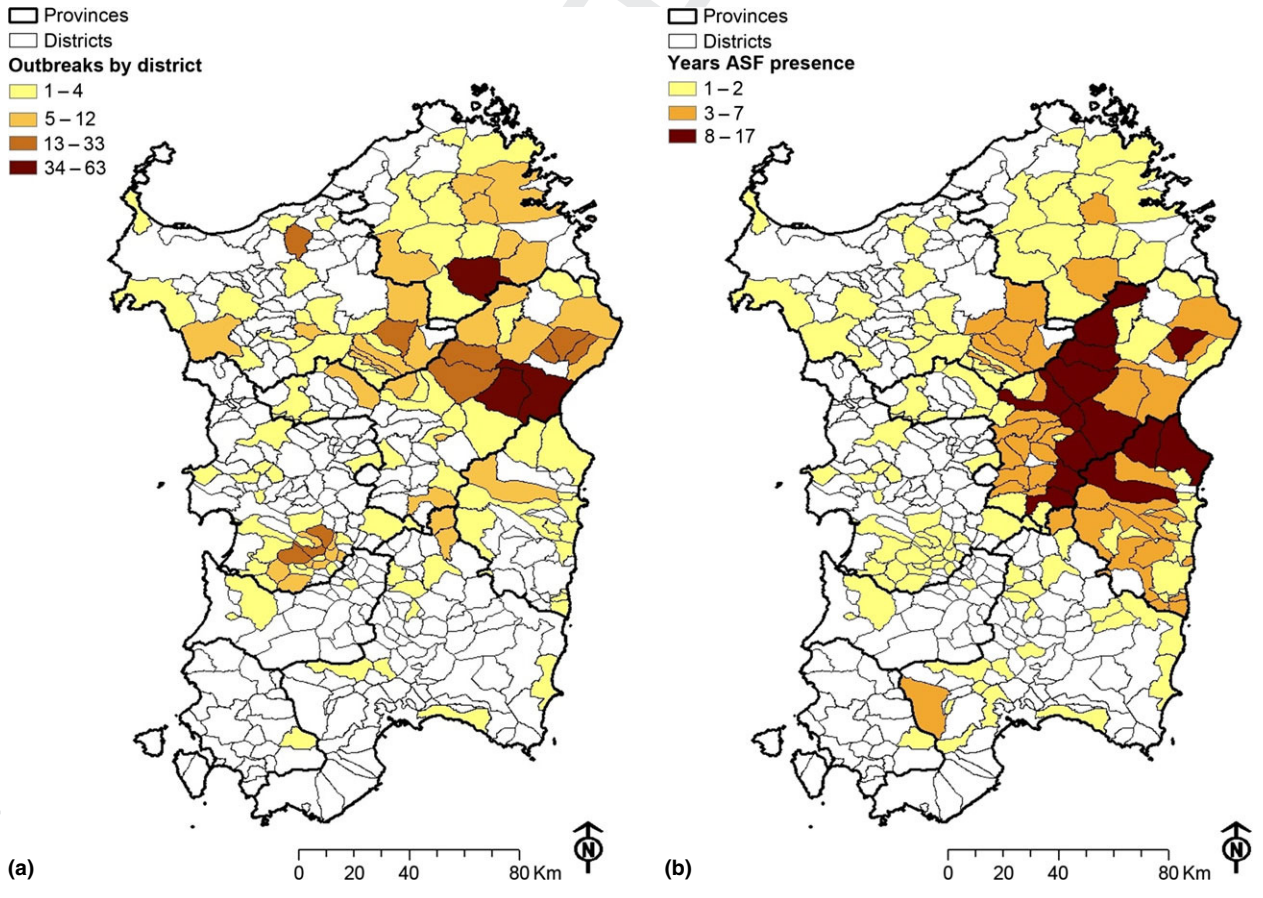

Fig. 5. Maps representing the evolution of African swine fever (ASF) from 1987 to June 2013. Number of ASF outbreaks per district (a) and number of years when ASF was declared in the district (b).

Table 1. Detailed information about the spatio-temporal clusters of the disease identified in Sardinia from 2004 to June 2013

Cluster number	1	2	3	4
Centroid district (comune)	Uras (Oristano)	Luculi (Nuoro)	Oschiri (Olbia-Tempio)	Burgos (Nuoro)
Latitude	39.71	40.42	40.7	40.37
Longitude	8.72	9.58	9.04	9.02
Radius (Km)	18.6	28.14	30.18	3.33
Start date	01/05/2005	01/04/2004	01/05/2013	01/06/2012
End date	30/09/2005	31/10/2004	31/12/2013	31/08/2012
P value	1,00E-17	1,00E-17	1,00E-17	4.9E-09
Expected outbreaks	29.43	59.50	39.89	0.35
Observed outbreaks	144	186	135	9

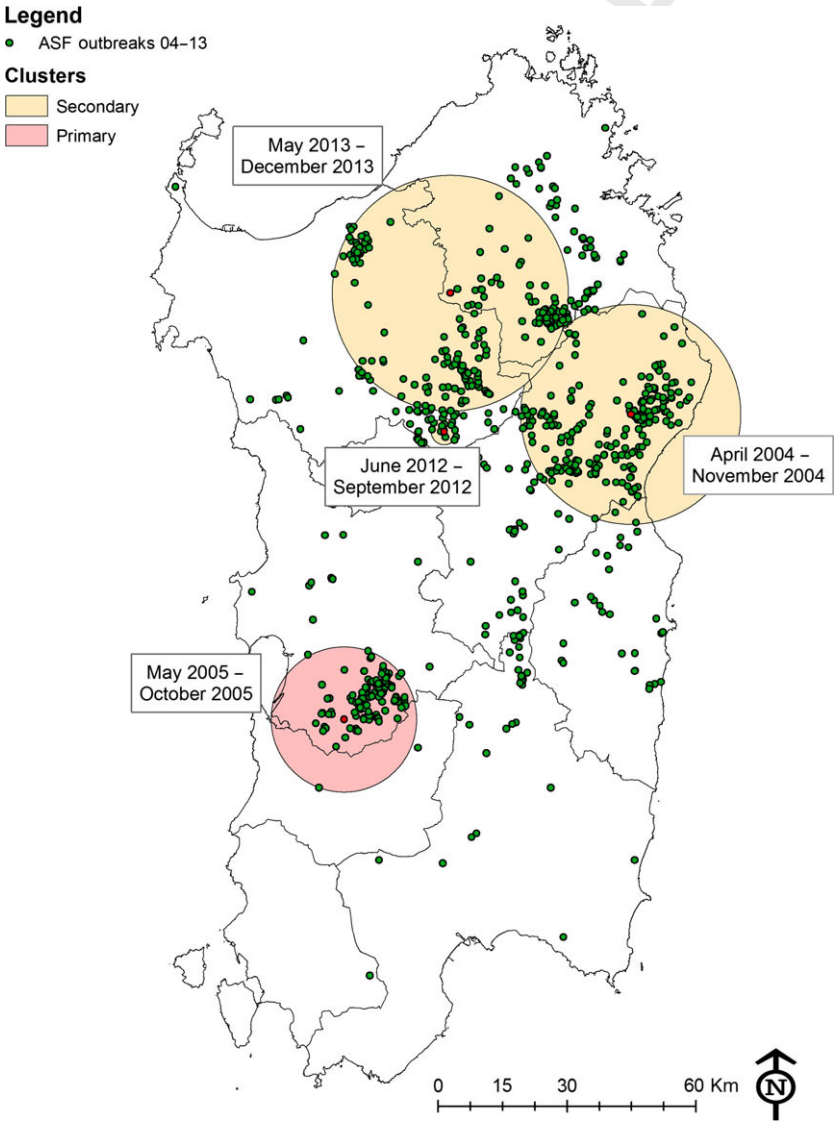

Fig. 6. Map of Sardinia with the outbreaks notified from 2004 to 2013 and the spatio-temporal aggregations identified by the cluster analysis.

the four identified clusters were located outside the endemic zone could be partially related to the lack of awareness, knowledge and/or good practices to control the disease in areas located outside the endemic zone. Potentially, once the disease enters one of these naïve zones, delayed or inadequate disease control could facilitate rapid disease spread,

and consequently, a large number of outbreaks could rapidly emerge in the same area during a short period of time (cluster). The presence of these new clusters could also be related to increased reporting when ASF is introduced to new areas, or to the higher pig density and greater trade relationships (i.e. faster disease spread), as compared with the endemic areas.

Factors that Make ASF Eradication in Sardinia Difficult

African swine fever has also been present in other European countries, such as Spain or Portugal, given similar or even more unfavourable environmental or socio-economic conditions than those in Sardinia (e.g. the presence of *Ornithodoros* ticks). However, with tremendous efforts, the disease was finally eradicated from all these territories, except for Sardinia. The exact causes of ASF disease persistence on the island remain unknown. The main explanations are likely related to sociologic and cultural factors, rather than pig susceptibility, environmental or biological reasons.

Non-professional pig producers (small and backyard farms)

As previously described, most Sardinian pig holdings have <15 pigs, meaning that the majority of holdings involve low-intensive production. It is important to differentiate between these small producers and the industrial producers which follow international breeding practices, good farming standards and strict biosecurity measures. The industrial sector has been significantly damaged by the presence of ASF in Sardinia; it has been suggested that the illegal practices of some of the non-professional pig producers increase disease risk of all.

For many of these small producers, pigs are a secondary income, and no investments are made to build holdings or purchase needed equipment (water supplies, disinfection devices, etc.). The typical structure of these small pig holdings involves nothing more than a mattress and some pieces of wood, with very limited to absent biosecurity and unhygienic conditions (Fig. 7). The administrative permits require that all holdings have fencing, yet pigs are sometimes housed with other species (sheep and/or bovines) and often have free access to pastures, both of which greatly increase the risk of disease spread.

The primary problem of the non-professional holdings is the lack of awareness and education among the farmers about animal husbandry, health and biosecurity protocols. Small-scale farmers are uninformed about the importance of simple biosafety measures such as the exclusive use of boots for the holding, disinfection procedures, control of the origin of feed and animals, quarantine procedures, not sharing equipment, etc. Educational campaigns have been initiated on the island to inform farmers about the disease; however, considering the current epidemiologic situation of ASF, they do not seem to have been fruitful.

Fig. 7. Picture of a traditional small-scale pig farm in Sardinia.

Colour online, B&W in print

Traditional free range

The tradition of breeding pigs in free-range systems is another important problem, which is deeply rooted in the culture of some parts of the island. It is a relatively profitable activity because there is no cost for feeding pigs and their meat is greatly appreciated in the region for its flavour, which is obtained from pasture fodder, acorns, chestnuts and other fruits. This practice is most often practised in economically deprived inland and mountainous areas with limited outside access and available pasture/grazing options from the land.

Traditionally, these pigs were bred in the communal areas belonging to the district or the city. In these communal areas, different animal species from different owners were brought together for several months of the year. From the very first epidemic, the practices of free-ranging animals, mixing animals and swill feeding was associated with a higher risk of ASF transmission. Since then, attempts have been made to regulate these practices by law, but often with little success. In the 1990s and the beginning of the twenty-first century, herders could breed pigs in these communal areas if they obtained a permit from the mayor of the city. However, since 2012, this practice is completely forbidden and all free-range pigs noted by forest guards or veterinarians should be notified, culled and destroyed under strict control measures. Unfortunately, free-range pigs are rarely identified and culled, and currently, it is still possible to find unidentified free-range pigs on roadsides or in forests (Fig. 8).

Colour online, B&W in print

Fig. 8. Free-ranging pigs on roadsides in Sardinia.

Illegal trade of products and animals

The illegal trade of products and animals frequently occurs. The existence of unidentified pigs is a common practice in certain areas of Sardinia, especially in the central-eastern part of the island. Piglets and products from these illegally raised pigs are sold and consumed, despite the lack of traceability and potential risk of the consumer. For example, the number of sows included in the 2012 census (NDb) does not match with the small number of slaughtered pigs registered (collected by IZS). This means that that many more pigs are produced than slaughtered under control, suggesting that irregular trade likely occurs in a given number of registered holdings. These illegal practices may be one of the several factors mostly contributing to the maintenance of ASF on the island (Martínez-López et al., 2011).

Other factors that challenge disease eradication

In addition to these socio-cultural factors, other epidemiologic features are likely involved in the persistence of ASF in Sardinia. The island's geography includes numerous natural resources, with thousands of hectares that remain unexploited by humans. The vast woodlands and Mediterranean forests allows for an abundance of wildlife species, including wild boar. This habitat, comprised of woodlands, scrubs and Mediterranean bush, makes the separation of metapopulations difficult (Aloi et al., 2007) and consequently facilitates the spread of disease between domestic and wild boar.

Numerous studies describe the role of wild boars in ASF epidemiology, suggesting that wild boars play a secondary role, and that they are unable to maintain the disease themselves. For example, outbreaks occurring in non-endemic provinces with no free-range practices (such as Carbonia-Iglesias), were successfully controlled 1 year after ASF outbreaks occurred in wild boars. However, interestingly, in certain areas of Sardinia, where wild boars frequently contact free-range pigs, their role may be crucial because of their density and frequent contact with free-ranging pigs. Hunting habits and practices can also greatly influence the spread of ASF: correct disposal of offal, animal sampling and compliance with the prohibition of hunting and may all help prevent disease transmission.

Although the climate and habitat of Sardinia favour the presence of ticks, as occurs in Spanish and Portuguese territories (Perez-Sanchez et al., 1994), the presence of 6 *Ornithodoros erraticus* has not been demonstrated in Sardinia to date. The presence of *O. maritimus* has been reported only on Isola la Vacca on the south-west coasts of Sardinia (Manilla, 1990), but its role on ASFV transmission has not been studied in experimental conditions (European Food Safety Authority [EFSA], 2010). These older studies involved searching for ticks directly in burrows and pig holdings, but new research and more advanced techniques should be carried out to confirm tick absence.

Another common risk factor for viral outbreaks is genetic modifications of the virus itself, which could change its behaviour. This has not yet been found in ASFV isolates present in Sardinia. The isolate currently on the island has an almost identical genetic profile as it did at the very beginning for the three regions of the genome analysed. The genetic analyses performed in 36 Sardinian ASFV isolates (1978–2009), following the standard genotyping procedures (Gallardo et al., 2009), revealed no differences in the sequences of the gene encoding the protein p54 and the partial gene of the protein p72. Only a slight variation in the number of tretramer acid repeats within the central variable region (CVR) of the B602L gene was found, revealing the existence of two different temporarily related subgroups. However, this change is considered a very small mutation, and as no changes were found in the other main analysed regions (p72 and p54), the close relationship between isolates is confirmed and supports the hypothesis of a single introduction of ASFV in 1978 (Giammarioli et al., 2011).

The influence of these numerous factors (wild boar, ticks, and modifications of the virus) in the persistence of ASF has not been clearly elucidated. However, the socio-economic and cultural factors that were present in the 1980s still remain today in some areas of the island, and it is very difficult to change human behaviour. Only the strict application of law, along with the use of new techniques to facilitate disease management, can help fight against ASF in these areas.

New Tools Available to Control the Disease

As ASF entered Sardinia, considerable progress has been made with diagnostic and other epidemiological tools to control the disease. African swine fever diagnosis has greatly evolved with the use of enzyme-linked immunosorbent assay (ELISA) test for the detection of antibodies, which allows for easy and rapid screening of large numbers of samples, and polymerase chain reaction (PCR) for the detection of DNA virus. The ELISA was implemented early in the control programmes of Sardinia, and currently, it is one of the core techniques used in the surveillance and eradication programme. PCR is routinely used for the detection of virus in blood and organ samples because of its rapid performance, high sensitivity and reliability, even in poorly preserved samples.

Recently, new techniques to detect ASF antibodies have been developed, which can be performed in the field and provide serological results in <10 min. These immunochromatographic devices have been validated using both field and experimental sera, and test performance has been remarkable with 99% specificity and 100% sensitivity (Perez et al., 2011). Moreover, they can be used with whole blood and potentially with exudates from organs (under evaluation), which proves most useful when sera are not collected under good conditions, as frequently occurs with sera from hunted wild boar that are commonly haemolysed. Current legislation dictates that all wild boars hunted in an infected area must be sampled and kept under surveillance until negative results are obtained from the laboratory. This situation poses a problem in terms of cost, time and potential risk that hunters will hide carcasses to prevent these inconveniences. This quick, easy-to-perform field technique offers an alternative that allows for immediate detection of ASF antibodies. If a carcass is found to be antibody positive, more samples should be taken (including blood and organs) to confirm results, but immediate destruction and burial should be performed to reduce potential disease risks.

Another innovative technique is the use of filter paper for collecting samples used for antibody or antigen detection (Fernandez Pinero et al., 2010; Uttenthal et al., 2013). This type of sample can simplify and reduce transportation costs (no cold chain requirements) and the risk of the viral spreading (smaller amount of infectious sample collected). Oral fluid is becoming a widely used sample to detect several viral swine diseases such as PRRS, PCV-2, Influenza, or even ASF (Mur et al., 2013). An additional benefit of this method is that it allows sample collection without bleeding the animal, which is very helpful for testing both free-ranging pigs and wild boars because they are very difficult to catch and manage.

Joint Strategy

While all the aforementioned techniques show promise, they will have little or no impact on the control programme without the approval and support of all sectors involved in disease management (Sanchez-Vizcaino et al., 2012). As mentioned before, the influence of socio-cultural and economic factors on the persistence of the disease in Sardinia is significant, and therefore, collaboration is a crucial component to successfully fight against the disease. After 35 years of ASF persistence in Sardinia, everyone seems complacent with the presence of the disease and thus, a drastic change in mentality is required.

First of all, the Sardinian pig sector needs to undergo major transformations and modernization. Unspecialized pig production (a small number of pigs for self-consumption) should be clearly differentiated and separated from professional production. All pig holdings, including small ones, should comply with minimum biosecurity, registration and control measures (e.g. veterinary control of slaughtering). Information campaigns about the legal and minimum requirements for registered pig holdings should be organized and enforced.

Furthermore, if the intention is to maintain traditional Sardinian pig breeding in a free-range system, which is a type of production that may have added value in terms of animal welfare and sustainable production, it should be clearly differentiated and legislated. Illegal production poses a tremendous risk and causes problems for the more well-established pig sector, and as such should be more strictly regulated. If farmers are interested in continuing with free-range techniques, they should redefine this type of production by establishing appropriate regulations that includes the registration, fencing and control of animals.

Farmers and pig owners should be educated on the importance of the role they play in disease transmission, as well as the negative economic consequences that ASF outbreaks have on the island, the country and the EU. Outreach campaigns should focus on disease awareness, economic impact, transmission routes and biosecurity concepts. These campaigns should be easy to understand, offered in common congregation areas (main squares, meeting point in villages, cafeterias, etc.) and offered in languages such as Italian and Sardinian.

Punishment for illegal actions should be strictly enforced, while incentives for farmers to improve holding structures or management actions should be offered. Recent campaigns to legalize holdings have been well accepted by the population (2,500 holdings opened in the first semester of 2013 versus the 500 holdings in 2011). Although these new holdings did not prove very useful in areas where free-range pigs are widespread (Nuoro and Ogliastra), it is always better to promote the existence of

registered holdings rather than illegally hidden holdings that are not officially controlled.

It is essential that official veterinarians consider their role in disease control because they are at the forefront of surveillance: visiting farms, assessing outbreaks and connecting the authorities with farmers. Veterinarians are also an example for farmers, so their attitudes on cleaning and disinfection are essential for demonstrating good practices. They should be strict in reporting any legal incompliance. Veterinarians must strive to understand how the virus was introduced at the holding, when it occurred, and who is responsible. This is no easy task considering the large number of outbreaks that have recently occurred (120 outbreaks in 2013) and the high level of endemicity in certain areas, but it is the only way to effectively fight against the disease.

Laboratory diagnoses are important not only to confirm the presence of the disease, but also to also obtain as much information as possible about ASF epidemiology. Linking these results with epidemiological investigations is vital to understanding disease movement. Consistent communication between all stakeholders involved is required: from farmers to ASL veterinarians to laboratory diagnosticians (IZS laboratories), to epidemiology researchers to regional authorities.

Conclusions

African swine fever has been present in Sardinia for more than 35 years. The persistence of the disease on the island is significantly associated with socio-economic, culture and traditional practices, which are especially present in certain areas of the island where the disease remains endemic. Although several factors make disease control extremely difficult on the island, the collaborative efforts of the authorities, farmers and veterinarians, together with new diagnostic and epidemiologic tools, will prove the only way to eradicate the disease.

Acknowledgements

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Combination of simulation and statistical modeling to identify factors contributing to ASF endemicity in Sardinia

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Running head: Risk factors for ASF control in Sardinia

Abstract

African swine fever (ASF), one of the major threats of swine industry, is currently present in many of Sub-Saharan countries, some East European countries, and Sardinia that remains endemic since the introduction of the virus in 1978 despite all the control programs implemented. Some factors were suggested to be involved in ASF permanence in Sardinia, including wild boar, the abundant of low biosecurity holdings and the presence of free range pigs. However, up to day their importance in ASF epidemiology was not elucidated.

The present work simulates the spread of ASF in the territory of Sardinia within the censed domestic pigs, using the data available on pig farms, pig movements, detection probabilities and control measures. In addition, logistic regression models were employed for determining the role of illegally raised pigs and wild boar density on the ASF outbreaks occurred in Sardinia between 2007 and 2013. The results of the spread model suggest a generally low impact of the outbreaks, with 1.7 infected farms on average per outbreak, mostly infected by local spread (99%). Results of the spread model were strongly correlated with the magnitude of the real outbreaks occurring in Sardinia during the study period (2007-2013), validating the utility of the model and the high influence of the domestic pig holdings characteristics in ASF spread.

When analyzing the occurrence of ASF, wild boar density and illegally-raised pigs were found to be significant for it, as the presence of these two factors increase the risk of ASF appearance in the area. The general model indicates a higher influence of the illegal pigs in comparison with wild boar. However, when very high densities of wild boar are considered (more than 8 heads/km2), its influence is much higher than illegal pigs.

The maps created with these results and the evaluation of the risk factors provide interesting information for control managements of ASF in Sardinia that could help to adapt and elaborate control plans adapted to each specific situation.

Key words: African swine fever, spread disease model, control measures, logistic regression model.

51 **Introduction**

52 African swine fever is one of the most complex and important diseases of swine that affects all
53 types and ages of swine. It is an infectious disease caused by a DNA virus, ASF virus (ASFV),
54 that induces high mortality rates in the affected population, leading to important economic
55 consequences (Sánchez-Vizcaíno and Arias, 2012). Consequently, it is a notifiable disease to
56 the World Organization of Animal Health (OIE), and its presence in one area implies immediate
57 trade restrictions. It was firstly described in Kenya in 1921 (Montgomery, 1921), and its
58 presence has been demonstrated in most of the Sub-Saharan countries since then. From
59 Africa, ASFV escaped to Europe in three occasions. Firstly, in 1957 ASFV reached Portugal but
60 was quickly controlled without further consequences. However, the second introduction that
61 occurred in 1960 near Lisbon (Portugal), implied enormous consequences affecting not only
62 Portugal, but also spreading to Spain. For more than 30 years ASF persisted in the Iberian
63 Peninsula, from where it escaped several times causing outbreaks in different locations (Arias
64 and Sánchez-Vizcaíno, 2002). Remarkable examples are those ASF cases in South and Central
65 America (Simeon-Negrin and Frias-Lepoureau, 2002; Tokarnia et al., 2004), as well as in some
66 European countries, including Sardinia (Italy). From all these territories, except from Sardinia,
67 ASF has been effectively eradicated (Costard et al., 2009).
68 In Sardinia, although the introduction of the disease occurred in the South, ASF quickly moved
69 to Eastern parts, where it established for more than 35 years. In some of these municipalities
70 ASF was reported for more than 15 years (Mur et al., 2014). Several specific characteristics of
71 the Sardinian pig sector, including the abundance of low-industrialized pig holdings with low
72 or null biosecurity and infrastructures, joint to the presence of illegal herds, non-registered in
73 the census, were pointed out as risk factors and potentially responsible of the permanence of
74 ASF in Sardinia (Mannelli et al., 1997; Mur et al., 2014). Other factors as the role of the wild
75 boar and the presence of ticks were also studied with no determinant results. Up to day, wild
76 boar were assumed to be not able to maintain the disease if they do not receive further

77 contamination from the domestic pigs (Laddomada et al., 1994; Mur et al., 2012; Rolesu et al.,
78 2007). Similarly, field studies were performed for collection *Ornithodoros* in pig holdings in
79 Sardinia with no results (Ruiu A, 1989). However, despite these efforts, the mechanisms of
80 spread and ASF reoccurrence are still not completely understood, and the fact is that ASF
81 continues spreading all over the island.

82 In the present study we used detailed data on pig farming and animal movements in Sardinia
83 to simulate the spread of ASF by the use of an adapted spatial and stochastic spread model
84 called Be-FAST (Martinez-Lopez et al., 2011). Results of this model were compared with ASF
85 epidemics from 2007 to 2013 for model validation and assessment of domestic pig sector
86 influence in ASF spread. In addition, logistic regression models were employed in order to
87 evaluate the influence of illegal pigs and wild boar density in the occurrence of ASF outbreaks.
88 The results of these analyses were used for creating maps of ASF risk of spread and ASF risk of
89 occurrence in Sardinia. These data provides interesting information for the risk managers in
90 order to adapt the control methods against ASF to the places and risk factors identified herein.
91
92 **Material and methods**
93 *Data*
94 Very detailed data on Sardinian *pig farming* was available for 2012, including the number of
95 pigs, the type of production (closed and semi-free conditions) and the geographical
96 coordinates of each censed holding. Sardinian' pig holdings were classified in four categories,
97 bearing in mind the characteristics of the pig sector in the island. This classification includes
98 semi-free holdings (those farms that allow pigs grazing in a fenced terrain within the holding),
99 industrial farms (more than 1000 pigs censed), family farms (closed farms with 4 or less pigs
100 censed), and regular closed farms (between 5 and 999 pigs). The *movements between pig*
101 *holdings*, to and from the slaughterhouses were also available for 2012, including the date of
102 the movement, the number of animals shipped, origin and destination of these movements.

103 *Outbreaks of ASF* were available from January 2007 to the end of 2013, including very detailed
104 information of the geographic position (GPS coordinates), number of animals affected,
105 affected specie, date of suspicious and date of confirmation. Due to the application of the new
106 ASF eradication program, since June 2012 all the pigs visualized in the field without the
107 appropriate identification need to be registered by the forest guards and communicated to the
108 competent authorities. The records of this data, including the location (GPS coordinates),
109 number of animals and type of *illegal animals* visualized, was also available from 2012 to April
110 2014. Finally, data on *wild boar density* in Sardinia was available from the Assesorato
111 all'Ambiente of Sardinia Region. This data reflects the estimated number of wild boar heads
112 per km^2.

113 *ASFV spread model*

114 For the simulation of ASFV spread, a spatial stochastic model, referred to Be-FAST (Between-
115 Farm-Animal Spatial Transmission) disease model was employed. This model was originally
116 developed for simulating the daily within- and between-farms spread of Classical swine fever
117 virus (CSFV) into a specific region (Martinez-Lopez et al., 2011). It has been fully validated
118 (Martinez-Lopez et al., 2012), and used in different environments ensuring the utility and good
119 performance of the model. Recently, it was adapted for simulating the spread of ASFV in
120 Bulgaria, by the modification of the required parameters that differ from both diseases
121 (Alkhamis *et al*., submitted for publication).

122 In the present study, some parameters of the model that are specific for Sardinia were
123 modified, including the classification of pig holdings and the probability of disease detection.
124 Considering that Sardinia is endemic for ASF since 1978, a good perception and knowledge on
125 ASF characteristics were assumed.

126 At the beginning of each simulation, all farms are susceptible to ASF infection. Each farm was
127 simulated to be infected 100 times, being the index case of this simulation. Then, the

128 transmission processes (within farm and between farm) started, and control measures are
129 applied (based on the EU legislation) from the detection of the disease.
130 The results of each simulation include the number and identification of the farms infected, the
131 number of infected animals, the length of the infection, the origin of the infection (movement
132 of animals, trucks, local spread….) and the cause of disease detection (surveillance in the
133 control areas, clinical signs, etc.). Considering the number of farms that each holding infect
134 during the 100 simulations performed, an infectious value (R0) was provided for each farm,
135 representing the risk of ASF spread from this farm. These values were used for constructing
136 the risk map for ASF spread.
137 *Evaluation of spread model*
138 For the evaluation of BeFAST model, its results were compared to the data obtained from the
139 real outbreaks occurred in Sardinia from 2007 to the end of 2013. For that purpose, firstly we
140 created a program in Matlab® for grouping ASF outbreaks based on space and time. A network
141 of outbreaks was created identifying clusters (epidemics) that contain outbreaks with similar
142 characteristics in terms of time and space. Specifically, a space of 3km was employed and a
143 time frame of 15 days. By this method ASF epidemics were identified and individualized. For
144 each epidemic, the first outbreak occurring was considered the index case, and the number of
145 secondary outbreaks was calculated for each index case.
146 In parallel, for each index case of ASF epidemics, we extracted the values obtained by BEFAST
147 (R0 and duration of the epidemic) for the same farm. However, when these outbreaks did not
148 match with censed farms (because they correspond with wild boar outbreaks or closed farms
149 not included in 2012 census) a density function of BeFAST was employed. Inverse Distance
150 Weight algorithm was used with a 2Km power (Kartsen et al., 2005b) in ArcGIS 10.2(ESRI®),
151 and raster value of the spatial location was used.

152 Finally, the BeFAST infectious values (R0) of these index cases were compared with the real
153 number of outbreaks involved in each epidemic. The average and the standard deviation of the
154 differences between both parameters were calculated and presented in the results section.

155 *Logistic regression models*

156 In order to study the influence of the proposed risk factors (illegal pigs and wild boar density)
157 in the ASF outbreaks occurrence, several logistic regression models were employed. For that
158 purpose, data values for each parameter were mapped and analyzed by the use of ArcGIS9.1
159 (ESRI®). Specifically, a Kernel density function was employed for the data on ASF outbreaks
160 (2007-2013) and illegal presence of pigs and rasters of $1km^2$ were created. Wild boar densities
161 were available in raster format, so no transformations were performed. Raster values were
162 extracted for each cell (24094 cells), in addition to the name of the municipality.
163 In the first model, a logistic non-spatial regression model was used, assuming the dependent
164 variable "outbreak" (yes or not) and as independent variables, the estimated illegal pigs
165 density and the wild boars raster value.
166 A second model was used to estimate the spatial influences (in this case influence of the
167 municipality). For that purpose, a logistic Bayesian spatial regression model (including random
168 effects) was used, including the same variables as in the previous regression model.
169 These two models (with and without spatial effects) were also employed to analyze the
170 specific impact that very high densities of wild boars had in ASF outbreaks occurrence.
171 Consequently, a high density of wild boar was assumed when raster values are above 7 (i.e, 8-
172 9-10), which involve less than 20 % of the island shape. Based on this, wild boar values were
173 categorized in 0 (low density of wild boar) and 1 (high density of wild boar). This data was
174 used to fit the previously described models, representing model 3 (non-spatial-high wild boar
175 density) and model 4 (spatial-high wild boar density).

176 *Risk maps*

177 The results of the ASFV spread model (number of farms that each index case infects in the 100
178 simulations infectiousness) were used for creating the map for the risk of ASFV spread. The
179 raster maps were created employing the IDW algorithm in ArcGis 9.3.1 (ESRI®). Cells of 1km^2
180 were selected with a power of 2 and a fixed radius of 3km was used.
181 The risk map of ASF occurrence was created employing the results of the logistic regression
182 models (OR), as weights for the different layers. Therefore, the function weighted sum of 9.3.1
183 (ESRI®) was employed to weight the raster files of wild boar density and illegal pigs, for
184 obtaining the final ASF risk map of occurrence.
185
186 **Results**
187 *Pig sector data, movements and ASF outbreaks in Sardinia*
188 In summary, more than 200,000 pigs were censed in Sardinia during 2012, registered in 15,700
189 pig farms. The most common type of holding is the familiar one (39%), followed by the regular
190 closed farms (35%) and semi-free holdings (26%). Industrial farms account for only 1% of the
191 total number of holdings. The density of pigs is generally low, with 539 pigs on average per
192 district.
193 The number of movements registered in Sardinia during 2012 between farms was 961. These
194 movements were originated in 337 different holdings, and involved the movement of 8406
195 pigs. Therefore, only 2 % of the censed farms move pigs to another farm during 2012.
196 From 2007 to December 2013, 573 outbreaks of ASF were registered in Sardinia. ASF
197 outbreaks were notified in all provinces of Sardinia, although a higher number is concentrated
198 in Eastern provinces (Figure 2). The occurrence of ASF strongly increased from the middle of
199 2011, leading to the appearance of more than 200 outbreaks in 2013. This date corresponds
200 with the implementation of the new eradication program in Sardinia.

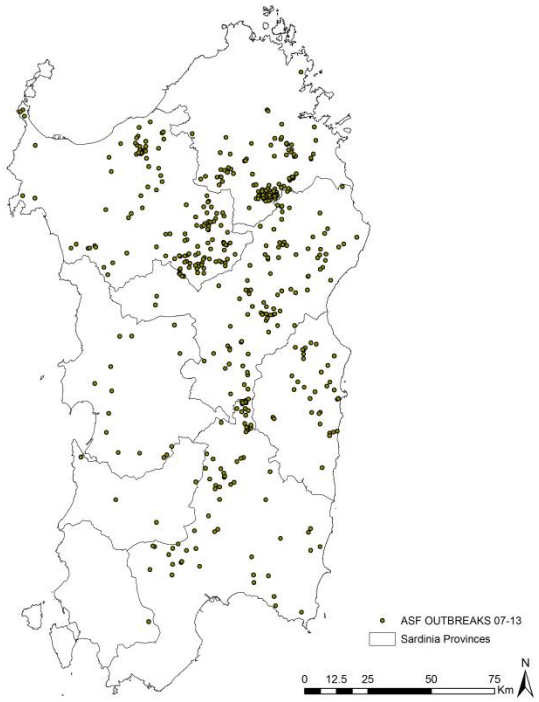


Figure 1: Location of ASF outbreaks occurred in Sardinia from 2007 to 2013.

ASFV spread model

The results of the simulation model indicated a general low potential spread of ASFV in Sardinia (average of 1.7 infected farms per simulation). 96% of the simulated epidemics infected less than 5 holdings, with a maximum of 70 farms infected. The average duration of the epidemics was 21.8 days with a maximum of 162 days (6.2 months).

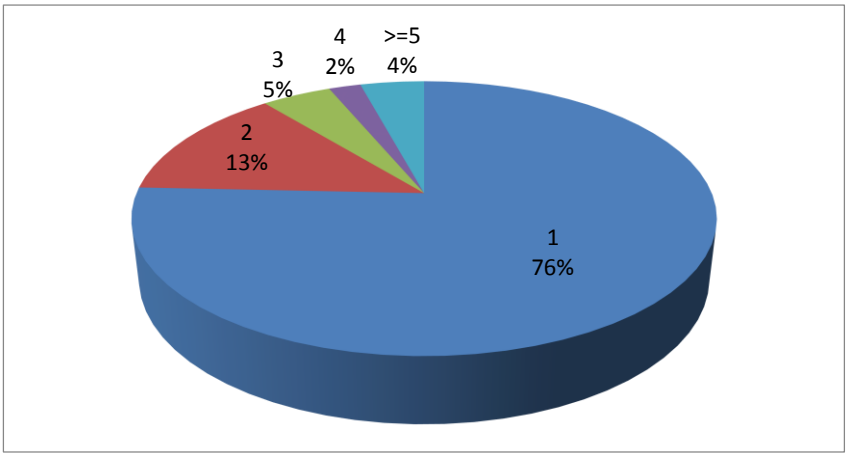


Figure 2: Percentage of simulations per number of farms infected.

Most of the secondary infections were caused by local spread (99%), whereas other sources of infection were very unusual (e.g. movement of animals and trucks). The detection of the disease most commonly occurred by clinical signs visualization (62%), followed by control actions in control areas (37%), surveillance and tracing activities (less than 1%).

On average, 40 farms were located within the control zone established in a radius of 3km around the outbreaks, and 230 farms on the surveillance zone (10 km radius). However, if the disease spread to further farms, it could involve a maximum of 534 farms within the control zone and 2381 within the surveillance zone.

Evaluation of spread model

The 573 ASF outbreaks occurred from 2007 to 2013 were grouped in 339 epidemics. The comparison of the extent of the real grouped epidemics with the estimated value by BeFAST, revealed a high similarity between both values. Without considering five epidemics that are clearly outside of the model, the average difference of infected farms between BeFAST results and the real situation was 0.2 with a standard deviation of 0.9. In general, BeFAST model predicts in a very reliable way the impact of ASF outbreaks in Sardinia (average deviation of one farm), although some bigger outbreaks were not explained by the model.

Logistic regression models

In all the models performed, both wild boar density and presence of illegally raised pigs were significantly and positively correlated with ASF occurrence in Sardinia (Table 1). Specifically, in the first two models, when using the raw wild boar density data, illegal presence of pigs presented higher OR (1.5 and 1.3) comparing with wild boar (1.2 and 1.1). However, when very high densities of wild boar were considered in the model (models 3 and 4), the OR of the wild boar (1.9 and 1.3) resulted higher than the OR for the illegal pigs presence (1.6 and 1.4).

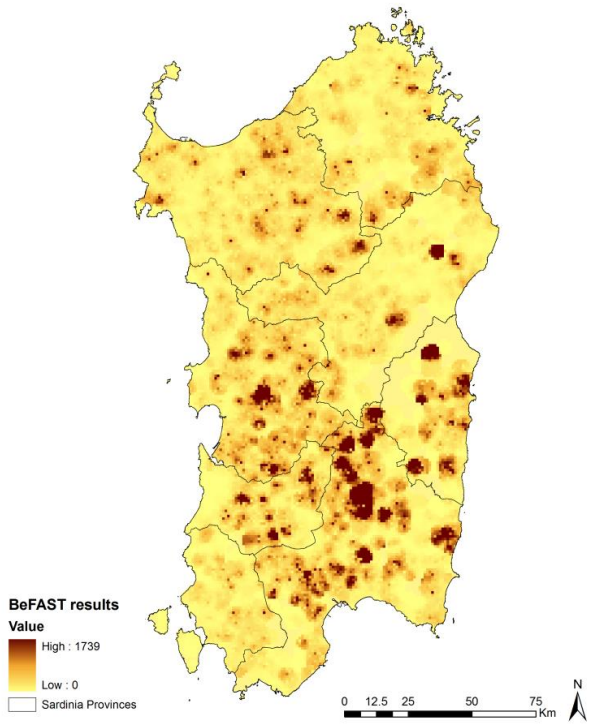
238 **Table 1**: Association values between wild boar density and illegal pigs, with ASF occurrence in
239 Sardinia. These are the results of the logistic regression model and Bayesian logistic spatial
240 model for the original data (models 1 and 2) and the high density wild boar data (models 3 and
241 4).

Variable	Logistic non-spatial model			Bayesian logistic spatial model		
	Odd Ratio	Conf.Int. (95%)	P-Value	Odd Ratio	Cred.Int. (95%)	P-Value
		Model 1			**Model 2**	
Wild boar	1.188	[1.172,1.205]	< 0.001	1.086	[1.061,1.113]	< 0.001
Illegal pigs	1.543	[1.460,1.632]	< 0.001	1.365	[1.220,1.528]	< 0.001
		Model 3			**Model 4**	
Wild boar	1.979	[1.854,2.113]	< 0.001	1.299	[1.170,1.442]	< 0.001
Illegal pigs	1.615	[1.528,1.707]	< 0.001	1.389	[1.241,1.554]	< 0.001

242 *Risk maps*

243 *Risk of spread*

244 The risk map created based on the potential infectiousness of each farms (Figure 3) shows a
245 general low risk of spread with special foci of higher potential for ASF spread. High risk areas
246 were identified in the Central-South of the island (north of Cagliari region), and Western
247 regions.



248

249 **Figure 3: Risk map for ASFV spread in Sardinia. Risk values represent the number of farms that each**
250 **farm could infect if it was potentially infected by ASF.**

Risk of ASF occurrence

Using the OR values obtained in all the logistic regression models, four risk maps were created for the probability of ASF occurrence in Sardinia (available as additional file 1). However, considering the strong similarity existing between all of them, only one map was included in the manuscript, specifically, the one obtained from results of model 1 (Figure 4). This map represents the areas with higher probability for ASF occurring due to the presence of wild boar and illegal pigs. As it can be observed in figure 4, the highest risk for ASF occurrence is located in the central East part of the island. In addition, other hot spots were located in northern regions, all of them areas with traditional presence of ASF.

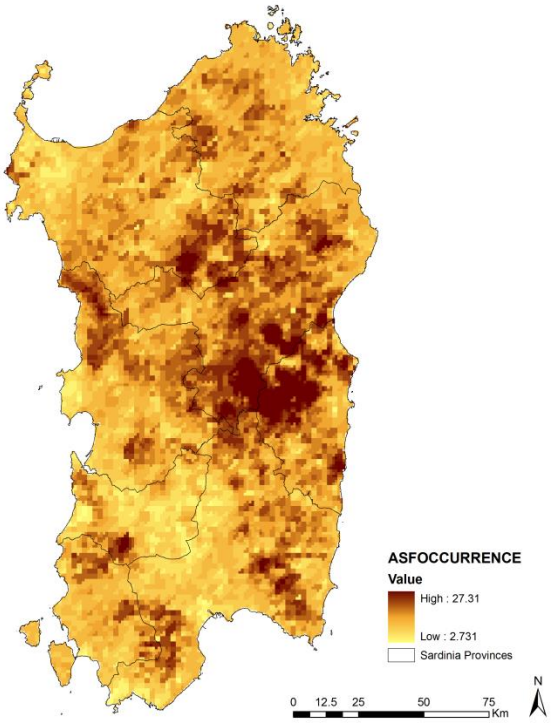


Figure 4: Risk map of ASF occurrence in Sardinia based on the results fo the logistic regression model.

Discussion

This is the first work using a combination of disease spread model and logistic regression model for identifying risk factors for ASF spread in Sardinia. The disease spread model

267 employed herein (BeFast model) was previously used in other scenarios for estimating the
268 impact of diseases outbreaks (Martinez-Lopez et al., 2011). However, in this case, its
269 application in an endemic area, allowed us to validate and compare the results with the
270 magnitude of the real outbreaks occurred in the past and consequently, formulate hypothesis
271 on ASF occurrence and spread.
272 The results of BeFAST indicate a generally low impact of the disease, clearly determined by the
273 low number of existing movements between farms. The low industrialization of the pig
274 industry in Sardinia seriously affects the model results, where most of the infections were
275 caused by local spread. On the other hand, considering the long time presence of ASF in the
276 island, it was assumed a high general awareness, and consequently, clinical signs would expect
277 to be detected quicker than in free countries. These assumptions had probably influenced in
278 the high proportion of outbreaks detected by clinical signs (62%), comparing with control or
279 surveillance actions. This situation clearly differs from the results obtained when applying this
280 spread model in other environments with a higher level of industrialization as Spain (Martinez-
281 Lopez et al., 2011).
282 The comparison of the model results with the data obtained from the real outbreaks revealed
283 that for most of the cases, BeFAST model predicts the magnitude of the epidemic with high
284 precision (average 0.2 and sd 0.9). Therefore, we can assume that this model estimates the
285 most probable occurrence of ASF that in general cause few secondary outbreaks, if any,
286 especially by local spread. This data could seem contradictory with the endemic presence of
287 the disease in the territory; but it perfectly matches with the reality of ASF in Sardinia, where
288 most of the outbreaks do not have a high impact. Some exceptions have been observed during
289 the last years in some northern and north-east municipalities, where ASF spread among 40
290 farms in a short period of time.
291 The model also indicates that the potential length and magnitude of the outbreaks depend on
292 the type of farm affected. This is highly significant in this territory where very different types of

293 production systems are present. In general, industrial farms have higher potential for infecting
294 other farms, as despite the higher biosecurity measures, the movements of animals and
295 fomites is much higher than in a small size farms. This fact allowed us to detect the farms with
296 high impact, and the areas where if the disease entered, would cause a big problem. The
297 results of the model also suggest that the most common route for ASF spread is by local
298 spread. Consequently, the strict application of biosecurity measures in infected farms, and the
299 reduction of personnel accessing to the farm could significantly reduce the magnitude of the
300 outbreaks. In addition, model results suggest, that in some areas ASF spread was very difficult,
301 leading to the appearance of more than 70 secondary outbreaks that persist for more than 6
302 months in the territory. Therefore, we can assume that as results of BeFAST model are
303 comparable with the reality of ASF in Sardinia (general small outbreaks with sporadic strong
304 epidemics), domestic pigs sector explains the spread of ASF from the occurrence of index
305 cases.
306 However, the continuous reoccurrence of the disease is not possible to be explained by a
307 spread model. Consequently, we decided to study the influence of two factors that have been
308 historically suggested to be risk factors for ASF in Sardinia. The role of the wild boar on ASF
309 epidemiology is controversial, as all studies performed so far suggest that their behavior to ASF
310 infection is similar to domestic pigs (Jori and Bastos, 2009). But, considering the high mortality
311 rates in this population, it seems that without any other source of infection, wild boar would
312 be not able to maintain the disease (Laddomada et al., 1994; Mur et al., 2012; Rolesu et al.,
313 2007).However, the recent introduction of the disease in Eastern EU countries by the
314 appearance of dead wild boars in the borders of Lithuania, Poland and Latvia, contradicts this
315 statement (OIE, 2014). In order to study their influence in ASF appearance in Sardinia, we
316 included wild boar densities in the logistic regression models. The second factor to be studied
317 is the presence of illegal pigs, that although are forbidden by regional authorities they are still
318 present in some areas of the island. This practice has been pointed out by several authors as a

319 risk for ASF (Mannelli et al., 1998; Mannelli et al., 1997; Mur et al., 2014), and sometimes has
320 been declared as the main problem of the island. The data on illegal pigs visualized in Sardinia
321 was used in the logistic regression models in order to study its correlation with the appearance
322 of ASF outbreaks.
323 The logistic regression model, both the non-spatial and the Bayesian one, when employing the
324 raw data on both parameters revealed that both of them are significant for the occurrence of
325 ASF outbreaks. Specifically, illegal pigs seemed to have a higher influence than wild boar.
326 Indeed, illegal pigs were supposed to be present in a 32% of the territory, where more than
327 48% of the outbreaks are concentrated. Therefore, the presence of illegal pigs in the territory
328 increases the probability of ASF occurrence.
329 In order to elucidate in detail the role of density of wild boar, we decided to categorize this
330 variable into high/no high densities. The results of the models using this categorized data
331 revealed that the role of wild boar at high densities is even more important than the presence
332 of illegal pigs. This led to the hypothesis that, the role of wild boar should be not studied with a
333 general approach, but considering the density of the population, and probably other factors
334 that affect their behavior as the habitat, feeding, water supplied, etc.
335 The final risk map for ASF spread identifies the areas of Sardinia where if ASF occurred, it
336 would spread more easily, causing higher negative consequences. In these areas, the quick
337 application of strict measures for disease control is fundamental for avoiding further
338 complications. Therefore, awareness campaigns and improvement of biosecurity conditions of
339 the farms located in this high-ASF spread-risk should be performed, even if they are not
340 located in the typical ASF endemic area.
341 In contrast, in the areas where the risk of ASF occurrence is higher, the control measures
342 should be focused in other terms, not only for the rapid control of the outbreaks, but also for
343 fighting against the factors that allow the disease to persist. Specific campaigns against illegally

raised pigs or for increasing biosecurity measures in very high density areas of wild boar (in order to avoid their contact with domestic pigs) should be implemented.

This work studied the determinants for ASF spread in Sardinia and the influence of two factors for ASF occurrence in the island. Authors are aware that some other factors are potentially involved in this complex problem, including illegal trade, cultural and sociological characteristics (Martínez-López et al., 2011; Mur et al., 2014). However, this work included the most important factors, providing important information for the design of specifically adapted control plans to the risk factor presence.

Conclusions

A disease spread model was applied in Sardinia from simulating the spread on ASF, revealing a general low spread among farms, mostly related with local spread. These results were compared with ASF historical outbreaks and seems to appropriately explain the magnitude of spread of majority of ASF outbreaks, whereas not all. Two factors were detected to be significant for ASF occurrence in Sardinia by the application of logistic regression models (Bayesian spatial and not); the presence of illegal pigs in the areas, and wild boar density. Specifically, when density of wild boar is considerably high, it strongly influences in ASF occurrence. The results and risk maps elaborated in this work would help to assess the importance of these risk factors and the necessity of implementing control measures adapted to each of them in the specific territories.

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ORIGINAL ARTICLE

Monitoring of African Swine Fever in the Wild Boar Population of the Most Recent Endemic Area of Spain

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Summary

Wild boars are natural hosts for African swine fever (ASF). The ASF virus (ASFV) can persist for long periods in the environment, such as in ticks and contaminated products, which may be sources of infection for wild boar populations. African swine fever was eradicated in domestic pig populations in Spain in 1995, after 35 years of significant effort. To determine whether ASFV can persist in wild boar hosts after it has been eradicated from domestic pigs and to study the role of wild boar in helping ASFV persist in the environment, we checked for the presence of ASFV in wild boars in Doñana National Park, one of the largest natural habitats of wild boar in Spain and one of the last areas where ASF was endemic prior its eradication. Samples from 158 animals collected between 2006 and 2010 were analysed using serological and nucleic acid-based diagnostic techniques recommended by the World Organization for Animal Health (OIE). None of the samples was found to be positive. These results confirm the absence of disease in wildlife in what was once one of the areas most affected by ASF in Spain, and they suggest that wild boars play a limited role in ASFV persistence. These results confirm that ASFV cannot persist in isolated wild boar populations for long periods of time without the interaction of other factors such as re-infection by contact with domestic pigs or by feeding on contaminated swill.

Introduction

African swine fever (ASF) is a haemorrhagic infectious disease that occurs in swine herds and causes significant economic and associated losses in affected countries. The disease first appeared in 1957 in Portugal, apparently introduced from West Africa. After a disease silence of 2 years, ASF re-emerged in Portugal in 1960 and spread to neighbouring Spain, where it remained endemic until the mid-1990s, affecting domestic pigs and Eurasian wild boar (*Sus scrofa*). Following tremendous economic losses, Spain implemented an effective and well-coordinated eradication programme in 1985 with $72 million of support from the European Community (EC) (Bech-Nielsen et al., 1995). Within the first 5 years, the programme achieved great success in confining the disease to southwest Spain (Andalucía and Extremadura) (Fig. 1; panels a and b). The virus persisted in these areas primarily because of (i) inadequate sanitary and biosafety conditions in outdoor pig production facilities, (ii) the presence of soft ticks (*Ornithodoros erraticus*), which serve as medium- and long-term reservoirs of the disease (Boinas et al., 2011); and (iii) the presence of an uncontrolled wild boar population, as was the case in Doñana National Park (DNP). Doñana National Park is one of the largest national parks in Spain and it is located in the Huelva province in the southwest of the country, one of the last regions to eradicate ASF because of the complex

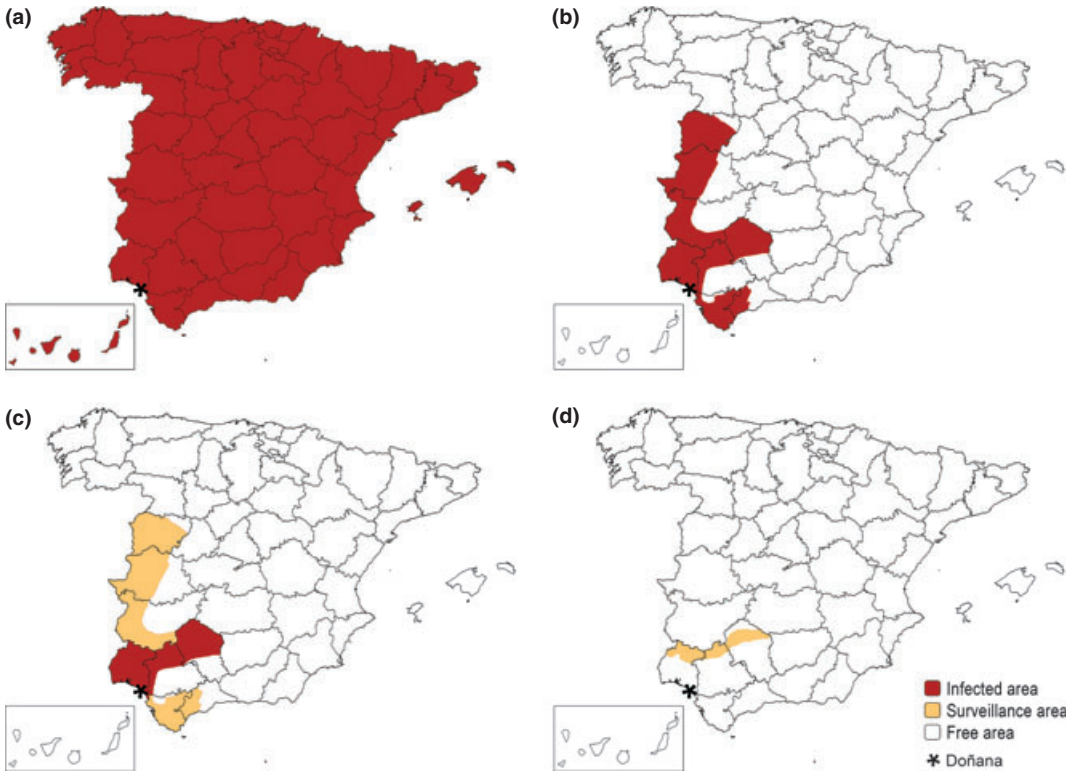

Fig. 1. Occurrence of African swine fever in Spain during the national eradication programme. (a) 1985, (b) 1989, (c) 1994 and (d) 1995.

epidemiology arising from the interaction between wild and domestic animals (Fig. 1). Serological monitoring carried out during the 1990s in the wild boar population in this region showed seroprevalence levels of 10% in areas where domestic pigs remained infected (Pérez et al., 1998). Moreover, 5.8% of ASF outbreaks in domestic pigs in Spain until 1981 were associated with potential contact between domestic pig populations and infected wild boars (Ordas, 1983). Within the DNP, in contrast to surrounding areas, there is no contact between domestic pig populations and wild boar populations, making this region suitable for testing for ASF virus (ASFV) persistence in natural hosts following the eradication programme in pigs.

No ASF outbreaks have been reported in Spain since 1994, confirming the effectiveness of eradication efforts. Nevertheless, ASFV can persist for long periods of time in the environment, soft ticks and contaminated products (EFSA, 2010). In particular, wild boars are a natural host for ASFV, as shown in studies in the Iberian Peninsula (Pérez et al., 1998), Sardinia (Firinu and Scarano, 1988) and in the currently affected areas of the Russian Federation and trans-Caucasus countries (TCC) (Beltrán-Alcrudo et al., 2009). However, their role in the persistence of the disease still remains unclear. Several studies have demonstrated that ASFV tends to disappear in wild boar populations when the interaction with infected domestic or free-ranging semi-domestic pigs is limited (Laddomada et al., 1994; Manelli et al., 1997, 1998; Rolesu et al., 2007; Jori and Bastos, 2009), suggesting that the virus does not persist for long in wild boars in the absence of other factors. This hypothesis should be tested more extensively, to gain a better understanding of the role of wild boars in disease persistence.

To confirm the ASFV-free status of a formerly endemic region in Spain and assess the role of wild boars in ASFV persistence, we conducted a wild boar surveillance programme in the DNP. Given that no ASF outbreaks have been reported in DNP or other parts of Spain since the eradication campaign ended, and based on studies of ASF in wild boars described previously, we hypothesized that even the large and dense wild boar population in the DNP, where ASF was widely present and persisted longest during eradication efforts, would be unable to maintain ASFV circulation. This hypothesis predicts that wild boars alone are unable to maintain the virus and do not interfere significantly with ASF control and eradication in affected countries as long as their contact with domestic pig populations is restricted.

Material and Methods

This study was conducted in the DNP (Fig. 1), where the estimated population of wild boar is 1700 animals (Equipo de Seguimiento de Procesos Naturales de la Estación Biológica de Doñana, 2011). This number was estimated by multiplying the animal abundance index obtained by DNP biologists, by the DNP surface (km^2). A total of 158 individuals were sampled during the hunting season from 2006 to 2010. Blood samples were collected from the heart or the thoracic cavity during field necropsies. Non-EDTA blood was allowed to clot, and serum was separated by centrifugation. Sera were sent to the ASF-OIE Reference Laboratory at the University Complutense of Madrid for serological and virological detection of ASF. The haemolytic or non-haemolytic status of sera was recorded before diagnostic procedures were performed.

Antibody detection against ASFV was performed using OIE-approved serological tests consisting of an initial screening of the 158 sera by indirect enzyme-linked immunosorbent assay (OIE-ELISA) followed by an immunoblotting assay (IB) to confirm doubtful and positive results. Briefly, the antigen in both conventional ELISA and IB assays was lysate from an MS stable monkey kidney cell line (ECACC, 91070510) infected with ASFV E70MS48, and the reporter system was protein-A conjugated to enzyme horseradish peroxidase (HRPO). Both procedures were carried out according to the OIE Diagnostic Manual (OIE, 2008) using ELISA plates, reference sera and IB strips supplied by the EU-ASF Reference Laboratory at the Animal Health Research Center in Madrid (CISA-INIA). A commercially available blocking ELISA (Ingezim PPA-Compac®; INGENASA, Spain) was also used to perform the initial screening.

African swine fever virus genome detection was performed on 146 sera, because the volumes of 12 samples were insufficient for DNA extraction. DNA was extracted directly from serum using the High Pure Extraction Kit® (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. DNA was amplified using OIE-recommended conventional and real-time PCR methods previously described by Agüero et al. (2003) and King et al. (2003).

FreeCalc v.2 software (Available at: http://www.ausvet.com.au/content.php?page=software) was used to test the alternative hypothesis that the wild boar population is free from the disease based on the results obtained with the sample in this study. The hypergeometric exact probability formula for imperfect tests and finite populations was used in the calculations (Cameron and Baldock, 1998). The chi-square test was used to determine whether an association existed between ELISA results and the haemolytic status of samples.

Results

Antibody testing was carried out on 158 sera, and 11 (7.0%) were positive by commercial ELISA and 38 (24%) by OIE-ELISA. These tests gave doubtful results for 6 (3.8%) and 29 (18%) sera, respectively. No positive samples were detected by IB assay. Because the OIE considers the IB assay as the confirmatory technique for detecting anti-ASFV antibodies, we concluded that no antibodies against ASFV were present in the analysed samples. None of the 146 samples analysed by PCR was positive by either the conventional or real-time method.

A total of 28 samples (18%) showed extensive haemolysis, while 49 (31%) showed moderate haemolysis. The chi-square test between OIE-ELISA results and haemolytic status of the samples showed a significant association between serum haemolysis and false positive results (P-value <0.001). This association was significant regardless of whether the extent of haemolysis was moderate or severe (data not shown). In contrast, the chi-square test for the association between haemolysis and INGENASA-ELISA results was not valid.

Discussion

This study aimed to verify the ASF-free status of the wild boar population in the DNP located in southwest Spain, the last region to achieve ASF eradication in the 1990s. All samples analysed were negative by both virus and antibody detection, suggesting the complete absence of the virus in the DNP wild boar population. Considering that (i) the total wild boar population in DNP has been estimated at around 1700 individuals; (ii) the sample size in this study was 158; (iii) the minimum number of wild boars to be sampled in the defined sampling area must allow for the detection of 5% seroprevalence with 95% confidence (Commission Decision 2003/422/EC); (iv) the sensitivity and specificity of the IB test are assumed to be at least 99% and 98%, respectively (Pastor et al., 1992); and (v) all samples tested were negative by IB and PCR methods, we can accept the alternative hypothesis that the population is free from disease at the 99.21% confidence level. Eradicating ASF in Spain was an extremely difficult and expensive process that required more than 30 years of efforts from the government, farmers and veterinarians. This tremendous work led to the development and improvement of the pig production sector in Spain, which has grown to 3.48 million tons per year, making the country the second largest pig producer in the European Union (Marquer, 2010). This massive growth could

never have been achieved with the production difficulties and international restrictions imposed on an ASF-infected country.

This study was carried out with the most reliable OIE-recommended techniques for ASF diagnosis. Nevertheless, the ELISA tests did give some false positive results. Specifically, the OIE-ELISA gave a higher number of false positive results when haemolytic samples were analysed ($P < 0.001$), revealing a problem of specificity in this test when wild boar hemolysate sera- such as the ones in the present study- are analysed. These results confirm previous findings that the OIE-ELISA lacks specificity and sensitivity when applied to poorly preserved samples (Gallardo et al., 2006; Perez-Filgueira et al., 2006; Gallardo et al., 2009) and supports the OIE recommendation of routinely confirming diagnostic results using the IB test (OIE, 2008). These results also highlight the importance of proper sample collection and preservation to ensure correct diagnosis, which is sometimes difficult when working with wildlife (Boadella et al., 2011). If samples have not been properly collected in a way that guarantees a minimum of quality and quantity, they should not be analysed, to avoid non-specific reactions and incorrect interpretations.

Major factors complicating ASF eradication in Spain included the low biosecurity level of the outdoor pig production systems at that time (1980s), the absence of an adequate identification and traceability system for pig herds and pig movements, and continuous contact between infected and susceptible domestic pigs, wild boars and soft ticks (Arias and Sanchez-Vizcaino, 2002). The last two factors (wildlife and soft ticks) are not easy to control, but must be considered in the design and development of disease control and eradication programmes. Infected wild boars posed a problem for the ASF eradication programme, specifically in parts of southwest Spain where outdoor pig husbandry methods with low biosecurity were used in areas with high wild boar density. During the last three decades, wild boars have expanded their range and increased their densities throughout the Iberian Peninsula (Gortazar et al., 2000; Acevedo et al., 2006). In fact, wild boars are considered overabundant in some circumstances, such as in fenced hunting estates where they are fed artificially as part of intense management, and in some protected areas where hunting pressure is low. These overabundant wild boar populations raise concerns about aspects of environmental conservation and disease maintenance (Gortazar et al., 2006). Wild boar densities in such areas can be as high as 90 individuals per km^2 and have been shown to correlate with infectious disease prevalence (Acevedo et al., 2007). In DNP for instance, *Mycobacterium bovis* infection prevalence grew from 33% to 52% between 1998 and 2007 (Gortazar et al., 2008). However, although European wild boars are highly susceptible to the disease and shed ASFV in similar quantities as domestic pigs (McVicar et al., 1981), their role in ASF persistence seems to be limited in the absence of infected domestic or feral pigs. This was the case in DNP, where no contact with domestic pigs is possible. Our study confirms that even a highly dense wild boar population, such as the one in DNP, cannot maintain ASFV circulation in the absence of other sources of infection.

Several Spanish regions recently initiated a wildlife disease monitoring programme that in 2011 was formalized into a National Wildlife Disease Surveillance Scheme (MARM, 2011). This Scheme proposes the sampling of 2070 wild boars across the entire country for surveillance of numerous wild boar diseases. In this study, we sampled three times more animals than the sample sizes of 58 and 60 animals required, respectively, by the National Scheme and by the European Community Decision 2003/422/EC. The purpose of this intensive sampling was to demonstrate the complete clearance of ASFV from wild boars at the highest possible confidence level.

Conclusion

The negative results of an ASF study within a representative wild boar population in Doñana National Park (Spain) support previous studies confirming that wild boars by themselves are unable to maintain ASFV infection for long periods. These findings also suggest the need to further study the role of these animals in ASFV persistence in currently affected areas, such as the Russian Federation.

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Conflict of Interest

The authors declare that they have no competing interests.

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OBJETIVO 4: Desarrollo de nuevos métodos diagnósticos para la vigilancia de PPA

La vigilancia es una parte esencial para la prevención y control de enfermedades. En el caso de la PPA, las muestras empleadas habitualmente para su vigilancia son muestras de suero y sangre analizadas por técnicas de ELISA y/o PCR. Esto requiere la toma de muestras de sangre de una gran número de animales, lo cual es un proceso laborioso, largo y muy costosos de por sí. En el caso de la PPA la recogida de sangre presenta un problema adicional, ya que el vPPA está presente en grandísimas concentraciones en la sangre de los animales infectados.

El objetivo de este estudio fue investigar el uso potencial del fluido oral como alternativa al suero para el diagnóstico serológico de PPA adaptando las técnicas ya existentes a esta nueva muestra. Con este objetivo, se tomaron muestras de fluido oral en distintos momentos post-infección de ocho cerdos inoculados experimentalmente con un vPPA atenuado. Estas muestras fueron analizadas empleando protocolos modificados de dos técnicas serológicas validadas, el ensayo inmuno-enzimático ELISA y la técnica de inmunoperoxidasa (IPT). Se detectaron anticuerpos frente al vPPA en las muestras de fluido oral en todos los animales desde momentos tempranos de infección, hasta el fin del experimento empleando tanto el ELISA como la IPT.

A raíz de este primer trabajo aquí presentado, hemos continuado trabajando con el fin de mejorar la sensibilidad de las técnicas diagnósticas para fluido oral. Los resultados de los nuevos estudios están en proceso de publicación, si bien ya han sido presentados en congresos internacionales, tal y como se detalla en el anexo 9.2.2.

Estos resultados confirman la presencia de anticuerpos frente vPPA en los fluidos orales de los cerdos, y abren la posibilidad de desarrollar programas de vigilancia basados en el diagnóstico serológico de fluido oral. Esta es una alternativa de gran interés, entre otros, para el estudio de poblaciones silvestres, siendo este uno de los problemas más importantes en la actualidad en el Este de Europa. En esta zona son necesarios métodos que permitan conocer el status sanitario de los jabalíes. El fluido oral podría ser la solución a estos problemas, si bien deberían adaptarse métodos para la atracción de los animales a los dispositivos, así como para la detección de virus por PCR permitiendo así una detección más temprana de la enfermedad.

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Potential use of oral fluid samples for serological diagnosis of African swine fever

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ABSTRACT

African swine fever (ASF) is a complex, highly lethal, notifiable disease of swine. ASF is wide-spread in sub-Saharan Africa and East European countries and there is presently a great risk of spread to neighboring countries. Since there is no vaccine for ASF virus (ASFV), control is based on rapid and early detection of the disease via surveillance. This approach requires collecting blood samples from large number of animals. Laborious and expensive of itself, this process also presents an additional risk because ASFV is present at high concentrations in the blood. The objective of this study was to initiate studies into the potential use of oral fluid as an alternative to serum for ASF diagnosis, for latter studying its possible use in surveillance and control programs. To this end, oral fluid samples collected at different times post infection from eight pigs experimentally inoculated with an attenuated ASFV were assayed using modified protocols of the two validated serological techniques, the enzyme-immune-liked assay (ELISA) and immunoperoxidase technique (IPT). Antibodies against ASFV were detected in oral fluid samples of all animals from early post infection through the end of the experiment by ELISA and IPT. These results confirmed the presence of ASFV antibodies in swine oral fluids samples, the possibility of an oral fluid-based approach in ASF diagnosis and, potentially in ASF surveillance.

1. Introduction

African swine fever (ASF) is one of the most complex infectious diseases of swine species. It is caused by a large doubled-stranded DNA virus, ASF virus (ASFV) belonging to the *Asfaviridae* family (Dixon et al., 2005). The epidemiology of ASF is complex, involving domestic and wild suids of different ages and breeds, as well as ticks from *Ornithodoros* genus. In affected countries, e.g., most of the sub-Saharan African countries, ASFV imposes a severe economic and social burden due to high mortality rates and international trade restrictions. In addition to Africa, ASFV is endemic in the island of Sardinia (Italy) and, since 2007, in a number of Eastern European countries, including the Russian Federation and the Trans Caucasian countries. In the recently infected areas of East Europe, ASFV's rapid expansion into north-western Russia demonstrated its capacity for rapid and far-flung spread, with numerous outbreaks occurring in such geographically distant regions as St Petersburg and the Baltic Sea, i.e., very close to European Union (EU) countries. The current situation in this region, in combination with its increased presence in African continent, highlights ASFV's potential for devastation and the risk it poses to the global pig industry (Sanchez-Vizcaino et al., 2012).

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There are no effective vaccines against ASFV. Therefore control is based on the implementation of strict prevention measures and the early detection of the disease, primarily through rapid laboratory diagnosis. At present, good antibody assays are available for the diagnosis of ASFV infection. These assays are suitable for use both in well-equipped international and national reference laboratories, as well as in basic regional and local laboratories. More recently, rapid assays have been developed for on-site ("point-of-care") use. Antibodies appear early in ASFV infection and persist for a long time, thereby serving as good markers of infection and providing for the detection of carrier animals. Antibody assays are economical, compatible with automation, and suitable for high-throughput screening. These factors make antibody testing the best option for ASFV surveillance (large scale screening) and eradication programmes (Arias and Sanchez-Vizcaino, 2002; 2012).

The limitation of this approach is the expense of collecting and testing blood samples. In particular, the need to handle and bleed animals presents a significant risk for further spread of ASFV as a consequence of the high levels of ASFV present in blood (McVicar, 1984). Oral fluid is an alternative diagnostic specimen that could potentially address these problems. Oral fluid has been proven to be a good diagnostic specimen for the detection of a number of pathogens of swine, including porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2), by both nucleic acid, and antibody detection (Prickett and Zimmerman, 2010; Prickett et al., 2011; Ramirez et al., 2012).

Given the current risk for the spread of ASFV, more cost-effective and rapid methods for ASFV surveillance are needed. Therefore, the objective of this study was to determine if antibodies against ASFV could be detected in oral fluid samples from experimentally infected animals as the first step in evaluating its potential use in ASF surveillance and eradication programmes.

2. Material and methods

2.1. Cells and viruses

A Spanish strain of ASFV isolated in 1970 (E70) and adapted to grow in a monkey stable (MS) kidney cell line (ECACC, 91070510) was used for OIE-antigen production following the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012). For the experimental in vivo studies, the attenuated and non haemadsorbing Portugal ASFV isolate NH/P68 (NHV) belonging to p72 genotype I and the virulent Armenia ASFV Arm07 isolate (genotype II) were used. Finally, isolate BA71V, recovered in Spain in 1971 and adapted to Vero cells (ATCC CCL 81) was employed for the production of the fixed indirect immunoperoxidase plates. The ASFV isolates were propagated and titrated according with Carrascosa et al. (2011). All ASFV isolates were obtained from the collections of the European Union Reference laboratory for ASF (CISA-INIA).

2.2. Experimental design and sampling collection

The ability to detect antibodies against ASF in oral fluid was evaluated using samples collected over time in two independent studies involving experimentally-infected pigs: (i) four Landrace × Large White pigs intramuscularly inoculated with 10^5 TCID50/ml of the ASFV isolate NH/P68 (NHV) and challenged at day 30 post infection (dpi) with 10 HAD50/ml of the heterologous virulent Armenia ASFV Arm07 isolate; and (ii) four Landrace × Large White pigs intramuscularly inoculated with 10^3 TCID50/ml of the attenuated and non haemadsorbing Portugal ASFV isolate NH/P68 (NHV). Paired oral fluid and serum samples were obtained at 0, 11, 14, 18, 21, 30, 37, 44, 52, 58 and 65 dpi(s). Both animal experiments were conducted at the BSL3 animal facilities at CISA-INIA and performed in accordance with the EC Directive 86/609/EEC, following the recommendation 2007/526/EC for the accommodation and care of animals used for experimental and other scientific purposes.

Oral fluid samples were collected from individual pigs using cotton rope 12 mm diameter and 25 cm length. Pigs were allowed to chew the rope for 10 min, i.e., until the rope was sufficiently wet. The wet end of the rope was cut, placed in a syringe (50 ml), and compressed to recover the oral fluid. A volume of ~5 ml was obtained from each animal on each collection day. Serum samples were collected using conventional methods.

2.3. ASF antibody detection in serum and oral fluid samples

ASFV antibody in serum samples was measured using the OIE indirect ELISA (OIE, 2012) and the Indirect Immunoperoxidase test (IPT). The IPT was performed using fixed VERO cells infected with isolate Ba71V ASFV following the same procedure described in COS1-cells by Gallardo et al., 2012.

The ASF ELISA and IPT serological tests were adapted to anti-ASFV antibody detection in oral fluid samples by adjusting incubation time, incubation temperatures, blocking buffers, concentrations of the antigen and the oral fluid samples, as well as the type and concentration of the conjugate (secondary anti-pig and/or protein A horseradish peroxidase [HRP] conjugated).

3. Results

3.1. Modification of OIE ASFV indirect ELISA and IPT protocols for oral fluid specimens

Optimization of the OIE ELISA and IPT tests for the detection of ASFV antibody in oral fluid specimens was carried out by comparing the responses of varying concentrations of antigen and conjugates using a checkerboard titration procedure. The optimum response for the indirect ELISA was achieved when microtitration plates were coated with 1 μg of ASFV cytoplasmic antigen per well, which correlated to a 1:800 working dilution. In addition, the OIE ASFV ELISA protocol was modified to enhance the detection of antibody in oral fluid, e.g., oral fluid samples were assayed without

dilution, the incubation time was increased to 16 h (overnight) at 4 °C, and protein A-HRPO conjugate was used at a 1:1000 dilution. Under these conditions, the optical density of the positive oral fluid samples collected late in the infection was approximately 10 times greater than the negative oral fluid samples collected prior to inoculation.

The conditions of the IPT test that optimized the positive/negative (P/N) ratio and produced the lowest background used oral fluid diluted 1:2 and protein A-HRPO diluted 1:1000. The IPT test was employed as confirmatory technique.

3.2. Comparison of oral fluid samples versus sera for anti-ASFV antibody detection

A comparison of the results of serum samples tested using the OIE-indirect ELISA, with the IPT used as a confirmatory test, versus oral fluid samples tested using the modified protocols showed that the levels of antibodies in serum and oral fluid samples followed a similar pattern, albeit oral fluid contained lower concentrations of antibodies. In the eight animals (C1 to C8) inoculated with the attenuated Portuguese isolate NHV/P68 (with or without further challenge), ASFV antibody was first detected in serum samples at 8 to 11 dpi(s) and, thereafter, through the end of the experiment. In oral fluid samples, ASFV antibody was first detected at 11 to 30 dpi(s) (Fig. 1A) and was detectable through the end of the study. Statistically significant differences were found in the level of antibodies of oral fluid samples by IPT analysis among the inoculated animals. The differences detected among the eight individuals were highly correlated with the serum antibody titers. In general, serum antibody titers $\leq$1:2500 were negative for ASFV antibodies in oral fluid.

4. Discussion

Cumulatively, the absences of vaccines, the early appearance and long-term persistence of ASFV antibodies, and the lower cost of antibody assays relative to PCR-based methods, justifies the crucial role of antibody detection to the control of the disease (Arias and Sánchez-Vizcaíno, 2012). In the present situation, there is a pressing need for more intensive and cost-effective ASFV surveillance, if further spread is to be avoided and control ultimately achieved. However, such efforts must be based on diagnostically effective, yet less costly, sampling procedures.

Previous studies have described the use of oral fluid samples as an economical alternative to serum for the surveillance of both human and swine diseases using either antibody and virus detection (Prickett and Zimmerman, 2010). Based on these reports, the objective of this study was to evaluate the feasibility of using oral fluid samples to determine the onset, level, and duration of antibodies against ASF from experimentally inoculated pigs. To this end, oral fluids from eight pigs inoculated with an attenuated ASFV isolate were tested using the optimized OIE-indirect ELISA and IPT tests. The results were compared to those obtained in standard serum samples analyzed by the validated OIE-indirect ELISA and IPT test.

The results revealed that antibodies against ASFV were detected in oral fluid samples from 11 to 30 dpi(s), depending on the animal, and maintained through the end of the experiment. The antibody response in oral fluid correlated with antibody titers in serum although a small delayed (median of 6 days) was shown in oral fluid samples of most of the infected animals. This fact could be explained by the lower quantity of antibodies present in

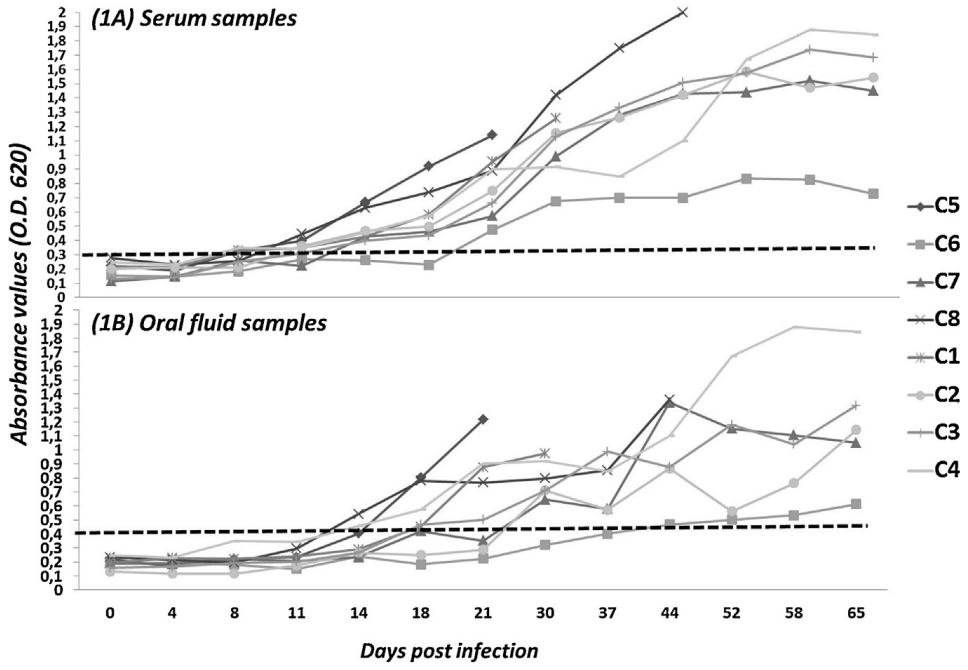

Fig. 1. Detection of ASFV-specific antibodies in serum (A) and oral fluid (B) samples using the OIE-indirect ELISA from eight pigs experimentally infected with the attenuated virulent ASFV Portuguese isolate (NHV). Cut off optical density value is represented by dots.

oral fluid compared with serum samples (Cameron and Carman, 2005). This lower concentration is responsible of the required adaptation of the diagnostic techniques, and nevertheless, results in a lower sensitivity of the test. The key issue for the future application of this sampling method is if it possible to assume this decrease of sensitivity, mainly referred to a delayed detection at early stages of infection. Future research should try to solve this problem, assaying other conditions for the assays, in order to achieve a similar sensitivity than with serum samples.

Future works should consider this option, as well as the use of a higher number of animals in the study. Authors are aware of the small size of this experimental study (8 pigs) in terms of statistical inferences and significances. However, our intention in this first approach was to elucidate if it was possible to detect ASF antibodies in oral fluid from infected animals or not; and for that purpose, a small group of animals was considered to be enough. This fact also explains the use of a non haemoabsorbing attenuated ASFV isolate (NHV) in the experimental infection. This ASFV isolate induces chronic clinical forms in infected animals which presents low viraemia in a late phase of infection (after 14 days post infection) combined with a high antibody responses developed at early stages (Leitão et al., 2001). This last point gave us the opportunity to study the antibody response through the time, trying to emulate a situation occurring in endemic areas with the presence of carriers circulating pigs, or in such places where moderate virulence ASF viruses are present, where the detection of antibodies are the recommended tests for the control of the disease. In these situations, oral fluid sampling should be considered as a potential non-invasive sampling method to be used as alternative in the control and eradication programmes in endemic areas.

However, this scenario differs from other situations occurring with virulent ASFV isolates, as those currently circulating in some parts of Africa and Russian Federation. Not because of the performance of the diagnostic techniques with different isolates (Gallardo et al., 2012); but for the acute form of the disease. In those cases, the problematic is the same with serum than oral fluid samples; the death of the animals could occur at very early stage of the disease, before the appearance of antibodies. However, even in those cases, it is important to no forget that the mortality of the virus is not always 100%, so oral fluid could be a good alternative for analysing the farms surrounding the outbreak, in surveillance programmes or checking the animals after the quarantine for antibody detection.

Overall, these data demonstrated that oral fluid could be used as an alternative diagnostic specimen for ASFV antibody detection using the modified ELISA and IPT tests. This study represents the first step of the research needed for the last goal of applying this test in ASF surveillance programmes. The use of oral fluid samples has many potential advantages in surveillance programmes, especially at this juncture in history when the risk for ASF introduction into the EU is high and targeted, enhanced surveillance is severely needed. In this study, oral fluid samples were collected from individual pigs in order to compare in parallel the results obtained in serum and oral fluid samples. However, as has been done elsewhere, future research should focus on pen-based samples, i.e., oral fluids collected from animals within the same pen, in order to study the detection of antibodies against ASFV in groups. Considering that the EU is free from ASFV (with the exception of Sardinia), and even more, that ASF is a mandatory notifiable disease, the detection of a single positive result implies immediate control actions. The use of pen-based oral fluid samples would obviate the need to differentiate individual animal status, thereby expediting the efficient detection of ASFV while saving money and time. A critical question related to this approach is the limit of detection, e.g. what percentage of pigs in a pen need to be ASFV-infected for the pen-based sample to test positive.

In addition, further studies will be required to fully validate this approach for ASF diagnosis in the field and its latter implementation in surveillance and control programmes. Further studies should increase the number of infected pigs, including a representative sample size and use other virulent isolate (e.g. the ASFV circulating in Russia) that develop an acute/subacute form of the disease. These future experiments would certainly clarify the potential application of this technique to the current situation in Europe, potentially including the detection of ASFV genome by PCR techniques. Based on the experience obtained during this experiment authors suggest to perform a daily collection of the samples until the first detection of antibodies, for the detailed analysis of the results and detection limits. It would be also interesting to include individual and group sampling in order to stablish the detection limits of the procedure. Finally, we also recommend to standardize and adapt the collection process to field conditions, fully accommodating it to farm structure, pig behavior, and the needs of specific disease surveillance programmes. It is important to note that the use of oral fluid samples always requires re-optimization of the assay because antibodies are present in oral fluid samples in lower concentrations than serum. Most serum antibody assays can be adapted to the detection of antibody in oral fluid by manipulating sample volume, incubation time and/or temperature, reagents, and cutoff threshold (Cameron and Carman, 2005), as it was done in this work for two serological techniques for ASF detection or in previous works and as was done previously for a commercial PRRSV antibody ELISA (Kittawornrat et al., 2012).

5. Conclusions

The results of this pilot study demonstrated the presence of antibodies against ASFV in oral fluid samples collected from infected animals at early stages of the infection. These data strongly suggest that oral fluid is a viable sample for the detection of antibodies against ASFV, potentially providing in the future a safe, efficient, cost-effective, and welfare-friendly alternative to serum for ASFV surveillance, control, and eradication programs. Such an approach would allow for fulfilling our responsibilities to animal health and commitment to animal welfare simultaneously.

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6. Discusión

Fuente: National Hog Farmer Association. http://nationalhogfarmer.com/

6. Discusión

Objetivo 1: Situación epidemiológica actual de la PPA e identificación de los factores que dificultan su control en el Este de Europa

La PPA es una de las enfermedades más importantes de cuantas afectan al ganado porcino, y así lo releja su pertenencia a la lista de enfermedades de declaración obligatoria de la OIE. En nuestro país y en Portugal, la PPA estuvo presente durante más de 30 años, durante los que se dedicaron grandes esfuerzos tanto en investigación básica del vPPA, como en diagnóstico laboratorial y diseño de planes para su control. De hecho, gran parte del conocimiento existente acerca de la PPA procede de aquella época, e incluso el programa de erradicación aplicado en España continúa siendo a día de hoy un ejemplo de éxito (Arias *et al.*, 2002). Sin embargo, desde que en 1995 la PPA fuese erradicada en Europa (a excepción de Cerdeña), la PPA pasó a un segundo plano a nivel internacional, reduciéndose los fondos y esfuerzos dedicados a ella. Durante esos años, otras enfermedades exóticas han acaparado toda la atención de la UE, destacando la PPC con sus numerosas incursiones en diversos países europeos incluyendo la afectación de los jabalíes, la fiebre aftosa ocasionando los brotes de 2001 y 2007 en Reino Unido o la afamada lengua azul y las polémicas asociadas a su vacunación.

Sin embargo, durante estos años de silencio aparente, la PPA ha continuado su avance, afectando progresivamente a un gran número de países africanos hasta ese momento libres de la enfermedad (Penrith y Vosloo, 2009). Son varias las hipótesis para este aumento de la incidencia en el continente africano, destacando el incremento de la producción de cerdo en el continente y la globalización de las comunicaciones, como los más plausibles (Costard *et al.*, 2009, Sánchez-Vizcaíno y Arias, 2012). Si bien, aunque no hay datos que lo confirmen, podría existir otro factor implicado, como es la aparición de cerdos de razas locales africanas asintomáticas portadoras de la enfermedad. Estos animales, cuya presencia ha sido citada por varios autores en diversos países africanos (Haresnape *et al.*, 1985, 1987, Mendes, 1994, Wilkinson *et al.*, 1988, Penrith *et al.*, 2004a), no muestran sintomatología tras la infección y podrían transmitir el vPPA y colaborar en la difusión de la enfermedad pasando totalmente desapercibidos. Bien sea por este motivo, o alguno de los anteriormente mencionados, el hecho es que actualmente en África **hay más países afectados de PPA que en toda la historia de la enfermedad**, existiendo mayor riesgo de difusión a territorios libres que en décadas anteriores.

Estos hechos pudieron favorecer la última salida del vPPA fuera del continente africano, ocurrida en 2007 a Georgia (Beltran-Alcrudo *et al.*, 2008). Desde allí, la PPA se fue difundió rápidamente a los países vecinos del Cáucaso como Armenia y Azerbaiyán, alcanzando Rusia en noviembre del mismo año por medio de jabalíes infectados. Tal y como se detalla en el objetivo uno de esta tesis, la PPA en Rusia siguió un avance continuo, estableciéndose en poco tiempo en la población doméstica del sur del país, donde permaneció presente durante los primeros años (2008-2010). En esta primera época se produjeron algunos brotes esporádicos en localizaciones muy alejadas de la zona endémica, normalmente por movimiento de productos contaminados, elevando las alarmas de todos los países colindantes.

Sin embargo, fue en el año 2011 cuando la enfermedad experimentó un cambio importante en su distribución que implicaba un importante riesgo para la UE y desencadenaría los hechos posteriores. Esta observación, realizada tempranamente en el artículo del objetivo uno (Sánchez-Vizcaíno *et al.*, 2013), ha sido posteriormente confirmada por otros autores, que consideraban a la región endémica de Tver como el mayor riesgo existente para la difusión de la PPA hacia territorios occidentales (Gogin A, 2013, Oganesyan *et al.*, 2013). Desagraciadamente, la enfermedad continuó difundiéndose, y en 2012 se declararon los primeros focos en Ucrania, seguido de Bielorrusia en 2013. Desencadenando estos hechos, finalmente, en la aparición de jabalíes muertos positivos a PPA en los territorios de Lituania, Polonia y finalmente Letonia durante 2014 (OIE, 2014b).

En el Este de Europa, la PPA ha establecido un ciclo epidemiológico en el que tanto el cerdo doméstico como el jabalí se encuentran implicados. Por el contrario, a día de hoy no se ha logrado demostrar la presencia e implicación de las garrapatas del género *Ornithodoros* en la zona (EFSA, 2010b). Esta situación podría ser bastante similar a la observada en España en los años 70 (a excepción de la implicación de la garrapata en España) o al ciclo observado en Cerdeña, lo que sin duda no constituye el ciclo más complejo de cuantos presenta la PPA.

Sin embargo, existen algunos **factores de riesgo puestos de manifiesto en esta tesis doctoral, que dificultan control de la PPA en la zona.** En primer lugar, Rusia está compuesta por un gran número de regiones, cada una de las cuales encargada de establecer e implantar el programa de control deseado para la lucha contra la PPA, ya que no existe un programa estatal de control de PPA. Esto implica que en dos territorios adyacentes, las medidas puedan ser diferentes. Del mismo modo, la financiación procede de nivel local y asociaciones de productores, por lo que no siempre las regiones pueden hacer frente al coste del proceso, y las compensaciones no están aseguradas para el ganadero. En determinadas regiones de Rusia, especialmente en el Sur donde la PPA

permanece presente de forma endémica, las granjas de traspatio son muy frecuentes, habitualmente presentando unos niveles de bioseguridad muy bajos. Este tipo de producción sumado a la falta de conocimiento sobre la enfermedad, favoreció la difusión y facilita la permanencia de la enfermedad. A su vez, el movimiento ilegal de animales y productos, el uso de los restos de comida para la alimentación animal, y la práctica de ocultar animales enfermos, son una realidad en la zona (Oganesyan *et al.*, 2013).

Todos estos problemas, sumados al desinterés tanto del estado como de los ganaderos en la implementación de las medidas (Gogin A, 2013), ha dado lugar a una adaptación a vivir con la enfermedad. Rusia ha aprendido a vivir con la PPA, y de hecho es el único país de la zona que, en vez de ver disminuida su población de cerdos, la ha visto incrementada, aprovechando la oportunidad para mejorar su industria porcina potenciando los sistemas industriales. La PPA se ha convertido en un problema social para los productores, pero no es un problema global para el gobierno ni la economía del país (Gogin A, 2013). Además, la mayoría de factores de riesgo identificados son de carácter socio-cultural y político, por lo que los cambios en ellos son muy difíciles y habitualmente se requieren varios años para observarse los efectos. Con todo ello, coincidiendo con otros análisis realizados (EFSA, 2014), consideramos de alto riesgo la probabilidad de que la PPA permanezca endémica en Rusia y los países del Cáucaso.

En este momento no es arriesgado afirmar que la PPA supone el problema y riesgo más importante para la industria porcina de la UE, y otras parte del mundo. Sin embargo, no debemos olvidar que la principal fuente de infección de PPA sigue estando en África y que este continente tiene cada vez más relaciones comerciales y empresariales con el resto del mundo. Un ejemplo de ello es China, que desde el año 2000 las importaciones y exportaciones de productos del continente africano se han multiplicado por 100. Estableciéndose además, importantes relaciones empresariales con numerosos países del continente, lo que implica un flujo continuo de mercancías y personas entre ambas localizaciones, y puede suponer un riesgo para la entrada de enfermedades, como la PPA (Sánchez-Vizcaíno *et al.*, 2014).

Por lo tanto, una vez asumido el importante riesgo que la PPA constituye para la UE, existen dos opciones para contrarrestarlo: prevenir su entrada en los países libres y, simultáneamente, controlar al máximo la enfermedad en aquellos países que está actualmente presente para reducir el riesgo en origen.

Objetivo 2: Caracterización del riesgo de introducción del vPPA en la UE para dos de las vías de entrada más importantes y desarrollo de una herramienta modular para la integración de análisis de riesgo.

En los países libres, teniendo en cuenta la ausencia de vacunas para PPA, las medidas de prevención se limitan a la implantación de medidas de bioseguridad estrictas que impidan la entrada de animales y/o productos contaminados. Sin embargo, es cierto que la aplicación absoluta de estas medidas en extensos territorios no es siempre sencilla, por lo que su eficacia puede resultar limitada dando lugar a la aparición de brotes. En la presente tesis se desarrolló un modelo de análisis de riesgo integrado que permitía relativizar los diferentes riesgos de entrada, señalando los países en los que el riesgo era más importante para cada una de las vías. Esta herramienta permitiría el diseño de planes de vigilancia de manera global para la UE, focalizando o incrementando las actividades de control en los puntos conocidos como de mayor riesgo. Para ello, primero se desarrollaron análisis de riesgo individuales para cada una de las vías. En esta tesis se recogen dos de las vías, cada una de ellas analizadas mediante el uso de una metodología distinta.

La primera de las vías corresponde con el riesgo de entrada de PPA a partir del comercio legal de cerdos vivos. Para esta vía existían datos suficientes relativos a las importaciones realizadas, por lo que se decidió emplear una metodología estocástica cuantitativa que permitiese estimar la probabilidad final de introducción para cada país durante el mes de análisis, siguiendo el esquema recomendado por la OIE (OIE, 2010). En este caso, los valores de riesgo obtenidos, al contrario que el resto de las vías (excepto importación legal de productos), fueron valores absolutos y no relativos. Los resultados de este análisis señalaron que la probabilidad anual de introducción de PPA en la UE por esta vía es muy baja ($5.22*10^{-3}$, lo que corresponde aproximadamente a un brote cada 192 años), por lo que no debería ser una de las prioridades a la hora de prevenir la entrada de la enfermedad. Estos resultados concuerdan con otros análisis cualitativos realizados para este riesgo (EFSA, 2010b, Wieland *et al.*, 2011, Gale *et al.*, 2010), que estimaron esta vía como de baja importancia para la introducción de PPA en la UE. El país de la UE que presentó un riesgo mayor para esta vía fue Polonia durante los meses de Noviembre y Diciembre.

Se evaluaron los resultados del modelo empleando dos bases de datos diferentes (EUROSTAT y TRACES), presentado un riesgo dos veces mayor el resultado obtenido con TRACES. Este hecho confirma, una vez más, la gran importancia de contar con datos de calidad y completos

para la realización de estudios epidemiológicos. Teniendo en cuenta que habitualmente los investigadores sólo tienen acceso a EUROSTAT (EUROSTAT, 2014), asumiendo lo observado en este estudio como frecuente, podría darse una subestimación del riesgo por fallo en los datos de origen. Para comprobar la influencia de este y otros parámetros empleados en el modelo, es fundamental realizar un correcto análisis de sensibilidad. En este caso se realizó un análisis de sensibilidad en dos pasos que confirmó la robustez del modelo empleado, detectando como parámetros más influyentes en los resultados finales, la probabilidad de supervivencia de los animales al vPPA, la prevalencia intra-rebaño y el número de brotes no detectados en el país de origen (Mur *et al.*, 2012b). Estos parámetros deberían ser considerados en el futuro, ya que si alguno de estos variase notablemente (e.j. aumenta la supervivencia de los animales al vPPA circulante), el riesgo final podría verse aumentado.

La segunda de las vías analizadas fue la del riesgo asociado a transportes (RAT) contaminados. En este grupo encuadramos tanto i) los camiones que podrían regresar de territorios infectados con PPA potencialmente contaminados (e.j. bien sea por la presencia del vPPA en las ruedas o en el interior de la caja), como ii) los restos de comida potencialmente contaminados que pueden traer los barcos internacionales, así como iii) los aviones internacionales, procedentes de lugares afectados con PPA. Estas vías habían sido señaladas anteriormente por otros autores como una de las rutas más importantes para la entrada de PPA en territorios libres (Costard *et al.*, 2009, Sánchez-Vizcaíno y Arias, 2012). Incluso, Alemania había analizado el riesgo asociado a los camiones de regreso; sin embargo, hasta la fecha el RAT global no había sido analizado (FLI, 2011).

En este caso, al no existir los datos necesarios para la realización de un análisis cuantitativo, empleamos una metodología semi-cuantitativa basada en el uso de estimadores de riesgo, combinados por ponderación con unos pesos obtenidos mediante criterio de expertos. Esta metodología es el resultado de la combinación de los métodos empleados en la modelización espacial de enfermedades (Stevens y Pfeiffer, 2011) con las estimaciones de riesgo basadas en opinión de expertos (Gallagher *et al.*, 2002, Gale *et al.*, 2010). Los resultados de este análisis no proporcionan un valor de riesgo absoluto, sino una estimación relativa para cada uno de los países analizados.

En esta vía hubo dos países que presentaron resultados de riesgo mayores, Polonia y Lituania, para las sub-vía de camiones de regreso específicamente. En general, el resto de países presentaron unos valores bastante bajos (16 de 27 países presentaron un valor bajo), si bien el

análisis detallado de los resultados muestra resultados interesantes que pueden ser consultados en la publicación. Un ejemplo es el valor relativo de alto riesgo que presenta Bulgaria para la sub-vía de movimientos de corta distancia en mar, claramente determinado por la presencia de los importantes puertos de Burgas y Varna en el mar Negro. De forma similar, los barcos de mercancías presentaron valores de riesgo elevados en Francia, Italia, Holanda y España, debido a las intensas relaciones comerciales existentes con Rusia, así como con países africanos como Nigeria y Angola. La sub-ruta, de las tres analizadas, que presentó un peso mayor fue la de los camiones de regreso contaminados (65% del total del RAT), seguido de los barcos y en menor medida los aviones (Mur *et al.*, 2012c). A pesar de la subjetividad implícita en los pesos asignados por los expertos, los resultados del análisis de sensibilidad mostraron una baja influencia de estos pesos sobre el resultado final del modelo, variando los valores de riesgo como máximo en una categoría.

El haber realizado dos tipos de análisis de riesgo empleando metodologías diferentes, permitió la comparación de los beneficios y desventajas de cada una de ellas a nivel usuario. Los modelos cuantitativos proporcionan valores de riesgo absoluto como resultado final, incluyendo a menudo parámetros de incertidumbre y gran detalle en las estimaciones. Esto permite una interpretación de los resultados más sencilla y directa de los resultados. Sin embargo, presentan importantes problemas, ya que para su realización se necesita gran cantidad de datos y mucho tiempo, esfuerzo y una base conocimientos estadísticos. Esto puede dar lugar a que cuando el análisis esté finalizado, el problema haya sido resuelto o la situación epidemiológica haya cambiado y sea necesario adaptar los parámetros epidemiológicos. Por el contrario, los análisis semi-cuantitativos son más subjetivos, no proporcionan un valor absoluto del riesgo y pueden dar lugar a malinterpretaciones y exceso de confianza en los resultados si estos no se interpretan adecuadamente (OIE, 2010). Sin embargo, si se seleccionan adecuadamente los estimadores y los pesos necesarios, y se elaboran con la transparencia y rigurosidad necesaria, son un tipo de análisis sencillo, con resultados fácilmente interpretables, capaz de proporcionar información clara, sencilla y de calidad para la gestión del riesgo.

Estas dos vías aquí presentadas, junto con otras tres vías analizadas en el marco del proyecto europeo ASFRISK (importación legal de productos cárnicos (Mur, 2010), entrada ilegal de productos (Costard *et al.*, 2013) y movimiento de jabalíes (laTorre *et al.*, 2013), fueron globalmente integradas en una herramienta modular. El objetivo de esta herramienta fue facilitar la comprensión y visualización de los análisis realizados, integrándolos en el mismo archivo con el

mismo formato y estructura, que fue proporcionada como material adicional junto a la publicación correspondiente. En el caso de los análisis semi-cuantitativos (RAT, ilegales y jabalíes) se incluyeron todos los cálculos, datos y elementos necesarios para calcular el riesgo asociado a estas vías. Por el contrario, para los modelos cuantitativos se incluyó una simplificación del modelo debido a la alta complejidad del mismo y la necesidad de emplear programas informáticos específicos para su visualización.

Los resultados de este análisis mostraron que un 48% de los países de la UE presentaban un riesgo relativo elevado (4 o 5 sobre 5) para alguna de las vías analizadas. La distribución del riesgo varió considerablemente entre las vías analizadas, ya que mientras que en la ruta asociada al transporte el riesgo se concentró en uno u dos países, en el resto de vías se repartió entre más estados. De hecho la media de países con un valor elevado de riesgo por vía es de 3,6 (Mur *et al.*, 2014). Esto indica la importancia de establecer planes conjuntos de prevención, involucrando a todos los países en situación de riesgo, con el fin de reducir el riesgo global.

El objetivo de esta herramienta, además de facilitar la comprensión del riesgo en ella calculado, fue el proporcionar una estructura base que pudiese funcionar a modo de plantilla para la estimación de otros riesgos. De tal modo, que si un país quisiese adaptar este análisis a sus diferentes provincias, solo tendría que emplear la estructura como plantilla y sustituir los datos de los países de la UE por los de sus provincias. Teniendo en cuenta la importancia de los análisis de riesgo y la seriedad y rigor con la que deben ser empleados, esta estructura constituye una propuesta que deberá ser siempre empleada por personal cualificado y con los conocimientos necesarios.

Del mismo modo, la interpretación de los resultados de riesgo continúa siendo uno de los mayores retos de los análisis de riesgo. En todos los análisis de riesgo aquí presentados, se estimó el riesgo de entrada de PPA en la UE, cubriendo así la primera etapa de liberación o introducción, sin considerar la exposición a este riesgo, siguiendo el esquema de la OIE (OIE, 2010). Esto implica que los resultados aquí presentados estimaron la probabilidad o riesgo relativo de que el vPPA alcanzase los distintos países de la UE por las distintas vías mencionadas. Sin embargo, el riesgo de que este vPPA ocasione un brote al entrar en contacto con la población susceptible está dentro del riesgo de exposición. Si el riesgo de exposición de mi población es muy bajo (altas medidas de bioseguridad, no se emplean desperdicios en alimentación animal, etc.), el riesgo final de introducción disminuirá notablemente a pesar de poder presentar un riesgo de entrada de vPPA elevado. Por lo tanto, los resultados de estos análisis de riesgo de introducción no proporcionan

una fecha exacta, ni aseguran que la PPA vaya a entrar por estas vías. Sino que sugieren los puntos críticos en los que la llegada de vPPA sería más probable, y por tanto, si no se establecen las medidas adecuadas, podría originar un brote de PPA. Ante estos resultados, los países con valores de riesgo elevados deberían preguntarse, ¿cuáles son mis medidas de control en estas vías? ¿Estoy convencido que aun entrando el vPPA por ellas, no entraría en contacto con la población susceptible? ¿Debería intensificar las acciones en alguna de ellas?

En la presente tesis se presentaron tres publicaciones de análisis de riesgo de entrada de la PPA en la UE que permitirán a los países que lo deseen, analizar los resultados del riesgo de entrada de PPA en su país y evaluar la necesidad de implementar medidas adicionales. Estas herramientas, además de tener una implicación directa sobre la prevención de la PPA en la UE, podrían ser implementadas tanto en otros territorios, como para el análisis de riesgo de otras enfermedades, mediantes las modificaciones necesarias.

Objetivo 3: Desarrollo de modelos para la evaluación de factores de riesgo para el control de la PPA en zonas endémicas

El control de la PPA debe basarse en aplicación de estrictas medidas de bioseguridad y el desarrollo de un plan de control que cuente con la participación de todos los sectores involucrados en el mismo (ganaderos, veterinarios, laboratorios, administración, cazadores, etc.). Existen unas medidas básicas, aplicables en todos los países; sin embargo, estos planes deberán adaptarse siempre al escenario concreto donde serán aplicados para garantizar su eficacia. No es lo mismo controlar la enfermedad en un país con una industria porcina desarrollada e industrializada como es la de España en la actualidad, que hacerlo en los años 60 cuando la PPA entró en nuestras fronteras. De hecho, curiosamente, los dos territorios donde la PPA está presente de forma endémica en la actualidad en Europa (Cerdeña y Rusia) presentan ciertas semejanzas epidemiológicas y socio-culturales con las de España en los años 60-70.

La propuesta del objetivo 3 de esta tesis es el estudio individualizado de los factores de riesgo implicados en cada escenario, para la consiguiente adaptación de los programas de control de PPA en la zona. De forma que, en base a esos resultados, se podrá dividir o planificar las fases del programa de control, estableciendo la localización y prioridades de las acciones a implementar.

Para ello, se tomó Cerdeña como ejemplo para la presentación de una metodología que permitiese priorizar factores de riesgo que permiten la difusión y permanencia de la enfermedad,

dificultando su control. Se realizó primero un estudio de la evolución de la PPA en Cerdeña con el objetivo de identificar los factores más influyentes que han podido propiciar la permanencia de la PPA durante más de 35 años. Se realizó una revisión detallada de la evolución de la enfermedad, analizando tanto la distribución como la temporalidad de los brotes y su asociación con los programas de control implantados en el momento. Los análisis demostraron que existe una clara asociación entre la presencia de la enfermedad y el terreno, existiendo municipios que han notificado brotes durante quince años seguidos, mientras que en algunas zonas los focos de PPA han sido nulos o esporádicos. Estas zonas consideradas tradicionalmente como la zona de riesgo hasta finales de 2011 (cuando el repunte de la enfermedad provocó la extensión de la zona de riesgo al territorio de toda Cerdeña), acumularon el 75% del total de brotes notificados desde 1987. Sin embargo, resulta curioso que en el de agrupación espacio-temporal realizado con los brotes ocurridos de 2004 a 2013, las agrupaciones más significativas no se localizaron en la zona tradicionalmente endémica, sino fuera de ella. Ello podría estar relacionado con que los brotes de PPA ocurridos fuera de la zona endémica tiene potencialmente un impacto mayor debido a la falta de preparación de los servicios veterinarios de la zona.

Cabe destacar que el sistema productivo en Cerdeña está bastante poco desarrollado, existiendo tan solo un 1,8% de granjas de tipo industrial, mientras que el resto son granjas la mayoría de pequeño tamaño, con escasas o nulas medidas de bioseguridad. En este contexto la PPA ha estado difundiéndose y moviéndose sin problemas. Otro factor señalado por algunos autores como el mayor riesgo para la PPA es la producción de cerdos en libertad, sin control alguno (Mannelli *et al.*, 1997). En muchos casos estos cerdos eran criados en tierras comunales compartidas por los vecinos del municipio, por lo que en ellas se concentraban animales de distintas especies y dueños sin control alguno. Esta práctica, a pesar de estar completamente prohibida a día de hoy, sigue existiendo en Cerdeña, especialmente en determinadas regiones de la isla (Figura 16).

Por el contrario, los jabalíes habían sido declarados por algunos autores como actores secundarios, los cuales sin la presencia del doméstico no eran capaces de continuar con la infección (Rolesu *et al.*, 2007, Laddomada *et al.*, 1994, Mannelli *et al.*, 1998, Mannelli *et al.*, 1997). A pesar de ello, es indiscutible que un cierto número de focos son detectados todos los años en la población silvestre, en la que el vPPA permanece presente desde el inicio.

Figura 17: Foto de un pasto comunal en Cerdeña, donde se observa la presencia de vacas y cerdos en libertad. Fuente: Imagen propia.

Tras este análisis inicial, con el objetivo de evaluar la influencia de los factores de riesgo identificados en la epidemiología de la PPA en Cerdeña, se desarrolló un estudio combinando un modelo de difusión de enfermedad con modelos estadísticos de regresión logística. Los resultados del modelo de difusión de PPA en doméstico revelaron una magnitud media de la epidemia de 1,7 granjas afectadas por brote, con un tiempo medio de duración de 18 días, siendo la ruta más frecuente de transmisión la difusión local. Estos resultados fueron bastante similares a las magnitudes de los brotes reales registrados entre 2007 y 2013, ya que los brotes en Cerdeña, quitando excepciones del año 2013 donde hubo grandes focos, suelen puntuales y de pequeña magnitud. Esto concuerda con el tipo de explotación mayoritaria presente en la isla, de pequeño tamaño con escasos o nulos movimientos y contactos con otras explotaciones. En estos casos, la única forma de reducir la difusión de la enfermedad es mediante la aplicación de estrictas medidas de bioseguridad que limiten o reduzcan la potencial difusión local por medio de visitas, personal veterinario, etc.

Por el contrario, para la aparición/ocurrencia de la enfermedad, tanto la presencia de jabalíes como los avistamientos de cerdos ilegales fueron factores significativamente relacionados. En general, la presencia de cerdos ilegales presentó una mayor influencia sobre la aparición de la enfermedad. Sin embargo, cuando se consideraron únicamente las densidades muy elevadas de jabalíes, este factor adquirió mayor peso que los cerdos ilegales. Estos resultados confirman la

importancia de la presencia de cerdos ilegales y altas densidades de jabalíes en la aparición de la enfermedad, mientras que las características y bioseguridad de las granjas domésticas son vitales para limitar la difusión de la enfermedad. A su vez, la creación de los dos mapas de riesgo permitió localizar las áreas donde la difusión de la PPA podría tener consecuencias más negativas, y donde los jabalíes e ilegales suponen un problema. Estos resultados permitirán priorizar los planes de control, en función de la presencia de uno u otro factor estudiado.

Además, los resultados obtenidos al analizar densidades de jabalí muy elevadas, revela la importancia de analizar en detalle la influencia del jabalí en la epidemiología de la PPA. En esta tesis se abordó este hecho desde dos puntos de vista diferentes, mediante el estudio de su papel para la permanencia de la enfermedad una vez desaparecido en doméstico (objetivo 3.2) o su papel en la reaparición de casos en un territorio endémico (objetivo 3.1). En cada caso se observaron resultados opuestos, de baja o alta importancia en función del evento a considerar (permanencia en ausencia de doméstico o aparición de la enfermedad). Por lo tanto, en posteriores estudios, se recomienda no generalizar sobre el papel del jabalí en el ciclo de PPA, sino estudiar cada caso en concreto, definiendo con detalle la pregunta a responder y estudiando los factores determinantes de la población de jabalíes implicada.

Los resultados de este estudio revelan que, mientras que en determinadas zonas de la isla las medidas deben enfocarse en el estricto seguimiento de las medidas de control y bioseguridad en las granjas de cerdos domésticos, en otras zonas los jabalíes y cerdos ilegales deberían ser la prioridad del programa de control. Se considera que el modelo de estudio propuesto en esta tesis podría resultar de utilidad para estudiar la influencia de factores de riesgo en otras zonas actualmente afectadas por la PPA, como podría ser Rusia o algún país africano, siempre y cuando hubiese datos disponibles para alimentar el modelo.

En paralelo, pero empleando un metodología diferente, se evaluó el papel de jabalí como reservorio de la enfermedad en una región de España. Para este estudio se recogieron muestras de jabalíes durante los años 2006-2010 procedentes del Parque de Doñana, siendo esta área donde mayores esfuerzos hubo que realizar para lograr la erradicación de la PPA. Además, en esta zona, se encontraban presentes varios factores de riesgo como son la presencia de *Ornithodoros*, alta densidad de jabalíes y producción e cerdo ibérico en extensivo. Todas las muestras analizadas resultaron negativas tanto a la detección de virus como de anticuerpos frente al vPPA, aunque algunas de ellas mostraron reacciones falsas positivas por la técnica de ELISA. Estas muestras fueron posteriormente analizadas por la técnica de Immunoblotting (IB), considerada por la OIE

como técnica de confirmación (OIE, 2013), dando todas ellas resultados negativos. Se evaluó la asociación existente entre el grado de hemólisis de los sueros analizados y la obtención de resultados falso positivos, obteniendo un valor significativo (p valor <0,001). Esto demuestra una vez más, la observación realizada por algunos autores de que la calidad del suero es fundamental para la obtención de resultados adecuados (Gallardo *et al.*, 2009b, Gallardo *et al.*, 2006). Esto es vital cuando se trabaja con animales silvestres, en los que frecuentemente, la calidad de las muestras no es la deseada (Boadella *et al.*, 2011). Por ello, el desarrollo de otros métodos de muestreo aptos para vida silvestre, permitiría la obtención de resultados más fiables.

Analizando los resultados obtenidos, el tamaño de la población en el área de estudio (Parque de Doñana) y el número de muestras (tres veces mayor de lo propuesto en el plan de vigilancia de Fauna silvestre), se considera a la población de estudio libre de la enfermedad con un 99,21% de confianza. Esto significa que 10 años después de haber sido declarada erradicada la PPA en la zona, la enfermedad no habría sido capaz de perpetuarse en la población de jabalíes, a pesar de las altas densidades allí observadas y la potencial presencia del vector *O.erraticus* en la zona. Estas observaciones coinciden con estudios anteriores desarrollados en Cerdeña (Mannelli *et al.*, 1998, Laddomada *et al.*, 1994, Rolesu *et al.*, 2007). El jabalí europeo sufre la infección de PPA de forma similar al cerdo doméstico y sus patrones de excreción son similares (Jori *et al.*, 2007). Las infecciones realizados con los aislados circulantes en el Este de Europa parecen señalar que presenta alta susceptibilidad, muriendo en periodos breves de tiempo (Blome *et al.*, 2013). Este hecho, sumado a las observaciones del presente trabajo y trabajos anteriores, reducen la importancia del jabalí como principal responsable de la persistencia de la PPA en el terreno.

Sin embargo, los hechos ocurridos recientemente en la UE, en los que los jabalíes han sido los primeros afectados y potenciales difusores de la enfermedad, nos invitan a pensar que su papel no debe ser del todo desestimado. A pesar de la ausencia de notificaciones al respecto, los hechos recientes parecen indicar que el vPPA está presente en la población de jabalíes de Bielorrusia. Ciertas informaciones no oficiales han comunicado el desarrollo de campañas masivas de caza en el país, lo que sin duda alguna ha propiciado el movimiento de los jabalíes, ocupando zonas fuera de su rango habitual de movimiento, alcanzado las fronteras de la UE. Esto pone de manifiesto la importancia del jabalí como vía de entrada de la enfermedad. Pero, ¿qué ocurriría si aislamos estas poblaciones de jabalíes del cerdo doméstico? ¿Acabaría desapareciendo la enfermedad?

Probablemente la respuesta a estas preguntas no sean una única sola, sino que dependerán en gran medida de las características de las poblaciones de jabalíes implicadas. Por ello

consideramos de gran importancia y actualidad el desarrollo de epidemiológicos más detallados que tengan en cuenta factores clave como la evaluación de las densidades animales, su hábitat, recursos alimenticios, presencia de puntos de encuentro, estructuras y grupos de animales, etc., y la relación de estos con el mantenimiento de enfermedades. Sólo así lograremos adquirir un conocimiento de utilidad que permita frenar el avance la PPA en la UE.

Objetivo 4: Desarrollo de nuevos métodos diagnósticos para la vigilancia de PPA

La vigilancia es una de las piezas fundamentales de cualquier plan para la prevención y/o control de enfermedades. Los métodos tradicionales de vigilancia activa se basan en la toma de muestras de un cierto número de animales para detectar la presencia o prevalencia de la enfermedad en cuestión. En el caso de la PPA, la serología ha sido la herramienta más empleada en los planes de control y erradicación para detectar los animales positivos y portadores, debido a su facilidad de aplicación a gran escala y bajo precio del test de ELISA (Arias y Sánchez-Vizcaíno, 2002). Esta técnica, al igual que la detección del virus por medio de la PCR requiere la toma de muestras de sangre por parte de un veterinario. Este proceso, además de ser costoso y lento, en el caso del vPPA conlleva un peligro asociado debido a la alta concentración de vPPA presente en la sangre de los animales infectados (Quintero *et al.*, 1986) .

Por ello, se decidió estudiar la utilidad de otras muestras para el diagnóstico de la PPA. En concreto, se evaluó el uso del fluido oral como muestra diana para el diagnóstico serológico de PPA.

Las muestras de fluido oral comenzaron a emplearse en veterinaria hace ya algunos años. Si bien su uso a nivel rutinario es bastante reciente, siendo el porcino la principal especie en la que se emplea (Prickett y Zimmerman, 2010). Existen varias enfermedades porcinas en las que el diagnóstico por medio de fluido oral está plenamente aceptado y estandarizado, existiendo incluso kits comerciales para la recogida del fluido y análisis de los mismos. Destacan para ello la detección de virus como el del Síndrome respiratorio y reproductor porcino (PRRS), el circovirus porcino tipo 2 (PCV-2), el virus de Influenza porcina o Torque teno virus (Ramirez *et al.*, 2012).

En esta tesis se evaluó el fluido oral como muestra de utilidad para la detección de anticuerpos en cerdo infectados con el vPPA. Para ello se llevó a cabo un experimento en el cual ocho cerdos fueron inoculados con un vPPA atenuado. Estos animales presentaron una sintomatología y curso de la enfermedad menos virulento que el ocasionado por un vPPA agudo, lo

que nos permitió realizar un mejor seguimiento de la respuesta serológica. Se tomaron muestras de forma paralela de suero y fluido oral a distintos días post-infección.

Los resultados obtenidos muestran que es posible la detección de anticuerpos frente al vPPA en el fluido oral, observándose una respuesta positiva a partir del día 11 pi en algunos animales, manteniéndose a los largo de la infección. Estos resultados están relacionados con la respuesta observada en el suero de los mismos animales. Sin embargo, para algunos cerdos concretos se observó cierto retraso en la aparición de anticuerpos (al día 30 pi) respecto al suero (Mur *et al.*, 2013). Nuestra hipótesis es que, en algunos casos, el retraso en la detección pudo deberse a fallos en la recogida del fluido oral. Al inicio del experimento se sangraba a los cerdos inmediatamente después de la toma de muestras de fluido oral mediante cuerdas. Esto hizo que los cerdos asociasen ambos eventos y algunos de ellos empezaron a rechazar el juego con la cuerda con el paso de los días. En estos animales reacios a jugar con la cuerda se intentó recoger cualquier contenido de la cavidad oral, incluso cuando el animal estaba dormido. Sin embargo, posteriormente observamos que para que la muestra fuese válida, el animal debe haber masticado la cuerda, no sirviendo el contenido de la cavidad bucal sin más. Esta primera experiencia nos sirvió para aprender y mejorar la técnica de recogida, haciéndola más atractiva y rutinaria para los animales. De este modo, se realizaron dos experimentos posteriores, en los que la toma de fluidos orales fue mucho más sencilla y efectiva.

Otro de los problemas observados referente a esta técnica es la ausencia de interés en los cerdos enfermos por la cuerda. En el caso de los vPPA de alta virulencia que cursan con un cuadro agudo acompañado de fiebre elevada y mortalidad repentina, esto supondría un verdadero problema. Los cerdos enfermos cuando presentan fiebre se muestran apáticos y rechazan cualquier actividad o juego con la cuerda, dificultando en gran manera la obtención de fluido oral. Por el contrario, esta técnica puede tener una gran aplicación en el caso de virus relativamente atenuados, o para el estudio de prevalencias y detección de animales portadores en situaciones endémicas. Por otro lado, la puesta a punto del uso del fluido oral como muestra diagnóstica para la detección del vPPA por medio de PCR facilitaría la detección temprana de la enfermedad, acortando los periodos detección. Por ello, en nuestro grupo hemos comenzado con los ensayos pertinentes para adaptar esta muestra a la detección del vPPA por medio de PCR, así como mediante Luminex®.

Del mismo modo, en nuestro grupo hemos continuado trabajando en la mejora de la sensibilidad de las técnicas diagnósticas para la detección de anticuerpos en fluido oral, obteniendo

resultados muy positivos. Para ello, en los ensayos posteriormente realizados, hemos sustituido el antígeno soluble del ELISA de la OIE (Sanchez-Vizcaino *et al.*, 1982), por antígenos recombinantes de ciertas proteínas del vPPA de importancia (vp72 y vp30). El uso de estos antígenos en los dos nuevos test de ELISA desarrollados nos han permitido alcanzar niveles de detección en fluido oral muy similares al suero, evitando así una de los principales problemas observados en el primer ensayo. Estos resultados han sido presentados en congresos internacionales (Giménez-Lirola *et al.*, 2014) y serán objeto de dos publicaciones científicas en proceso de elaboración.

El estudio recogido en esta tesis demostró la presencia de anticuerpos frente al vPPA en el fluido oral de animales infectados, confirmando la utilidad del fluido oral como muestra para el diagnóstico serológico de PPA. Para ello, se adaptaron dos técnicas diagnósticas (ELISA e IPT), con el objetivo de poder ser empleadas en los programas de vigilancia de PPA en países endémicos. La vigilancia de jabalíes es otro de los campos en el que el fluido oral podría aportar soluciones más interesantes. Considerando la situación actual de la PPA, con casos de jabalíes positivos al vPPA en tres países de la UE, los planes de vigilancia de poblaciones silvestre son una prioridad absoluta. Todos los animales hallados muertos deben ser analizados, pero también un número importante de los abatidos. En estas condiciones, el fluido oral al ser un método de muestreo no invasivo, permitiría conocer la situación sanitaria de los jabalíes sin necesidad de cazarlos, evitando el peligro que esta supone para la difusión del vPPA. Del mismo modo, en ciertas circunstancias en las que el manejo de los animales no es sencillo (véase animales semi-salvajes o criados en extensivo), esta técnica podría resultar ideal para el muestreo de los mismos. Es cierto que este último tipo de cerdos no está tan aburrido como el cerdo doméstico alojado en granja. Pero el uso de determinados atrayentes (azúcar, zumos, sabores y olores) podría motivar el juego con la cuerda. De hecho, en nuestro grupo se han realizado estudios preliminares con los jabalíes, observando una respuesta muy positiva.

En definitiva, el fluido oral es una muestra no invasiva, que respeta el bienestar animal, cuya recogida es fácil y sencilla y puede ser realizada por cualquier persona sin necesidad de cualificación sanitaria. Por lo que su uso en el diagnóstico de PPA, mediante la adaptación necesaria de las técnicas diagnósticas, se espera resulte en una mejora tanto de la eficacia y coste de la vigilancia de la enfermedad en animales portadores y supervivientes en zonas endémicas de PPA.

Los estudios desarrollados en esta tesis proporcionan soluciones y respuestas a la difícil situación que presenta actualmente la PPA. La revisión inicial de la epidemiología de la enfermedad, especialmente el estudio de su evolución y difusión en el Este de Europa, nos demostró la inmediata necesidad de estar preparados y prevenidos frente a una posible entrada, tal y como ha acabado sucediendo en algunos países de la UE. A su vez, la identificación de los factores de riesgo presentes en el Este de Europa nos permitió evaluar la situación y estimar la alta probabilidad de endemicidad en la zona, ya que existen muchos factores de difícil solución.

Teniendo en cuenta la presencia de este peligro y el gran impacto que la entrada de este virus podría ocasionar en la UE, se desarrollaron análisis de riesgo con el fin de señalar las vías y países donde la introducción de PPA sería más probable. Para ello empleamos diversas metodologías en colaboración con otros grupos de investigación, dentro del marco del proyecto europeo ASFRISK. En esta tesis se presentaron dos de estas vías, así como el resultado final del análisis, compuesto por una herramienta modular para la integración y visualización de los datos. El objetivo de estos estudios fue el de proporcionar a los países miembros una herramienta que les permitiese conocer el riesgo de entrada del vPPA por cada una de las vías y actuar en consecuencia a estos riesgos. A nivel comunitario, esta herramienta también serviría para señalar las rutas en las que la colaboración y esfuerzo mutuo sería necesario.

No obstante, no basta con prevenir la entrada, ya que sino eliminamos la fuente de origen de la infección (véase Rusia o Cerdeña), nos enfrentaremos a introducciones repetidas, algunas de las cuales podrían resultar en consecuencias muy negativas. Por ello, en esta tesis se desarrollaron herramientas para el estudio de factores de riesgo en zonas endémicas de PPA que permitiesen localizar y priorizar los planes de control en función de los factores existentes. Estos análisis desarrollados tomando Cerdeña y España como escenarios, podrían emplearse en otras regiones endémicas para PPA con el fin de obtener información de interés para el diseño de los planes de control.

Por último lugar, se detectó por primera vez la presencia de anticuerpos en el fluido oral de animales infectados, adaptando además dos técnicas serológicas para este fin. Este descubrimiento abre paso a una nueva muestra disponible para el diagnóstico de PPA, que un futuro podría implementarse en los programas de vigilancia serológica. Su uso en las zonas actualmente afectadas en el este de Europa (Rusia, Bielorrusia o Ucrania, o países de la UE) permitiría conocer los niveles de prevalencia de la enfermedad y tomar las medidas de control oportunas en cada

caso. Del mismo modo, su utilización en territorios endémicos como Cerdeña permitiría la delimitación de la zona infectada, asegurando la ausencia de enfermedad en zonas libres.

En definitiva, los trabajos desarrollados en esta tesis tienen como objetivo proporcionar información y herramientas que sirvan para i) mejorar los planes de vigilancia y prevención en la UE en base a los resultados de los análisis de riesgo, y evitar así la entrada de PPA; ii) identificar y localizar los factores de riesgo presentes en zonas endémicas para la consiguiente adaptación de los planes de control y erradicación y ii) facilitar el diagnóstico y vigilancia serológica de la PPA mediante el empleo de fluido oral como muestra diagnóstica. Se espera que los trabajos y resultados de esta tesis resulten en un beneficio común, proporcionando estrategias de utilidad en la prevención y lucha frente a la PPA.

7. CONCLUSIONES/CONCLUSSIONS

Fuente: Manual on African swine fever for pig producers. FAO, 2001.

7. CONCLUSIONES/CONCLUSSIONS

I.

Se identificaron como factores de riesgo que dificultan el control de la PPA en Rusia y países del Este de Europa, los siguientes: la ausencia de un programa de control coordinada y financiado a nivel estatal, la abundante presencia de cerdos de traspatio y otras producciones de baja bioseguridad, el comercio ilegal de cerdos y productos y el frecuente uso de desperdicios para la alimentación animal.

Following risk factors were identified in Russia and other East European countries as a difficulty for controlling ASF in the area: the absence of a coordinated and funded national control program, the abundant presence of back-yard pigs and other production systems with low biosecurity standards, and the frequent practice of swill feeding.

II.

Se ha evaluado el riesgo de introducción del vPPA en la UE por medio del comercio legal de cerdos vivos, existiendo actualmente una baja probabilidad de introducción por esta vía ($5.22*10^{-3}$, que corresponde aproximadamente con un brote cada 192 años). Por lo que esta vía no supone una prioridad actual en los programas de vigilancia y control de la PPA en la UE.

The risk of ASFV introduction into EU through legal imports of live pigs was evaluated, resulting in a low probability of ASFV introduction by this route ($5.22*10^{-3}$, which corresponds with one outbreak every 192 years). Therefore this route is not considered a current priority for ASF control and surveillance programs in the EU.

III.

El riesgo de introducción del vPPA en la UE por medio de fómites asociados a transportes contaminados se concentró principalmente en Polonia (4.13/5) y Lituania (3.8/5). Este riesgo está asociado mayoritariamente a los camiones de regreso potencialmente infectados (65% del riesgo por esta vía), seguido en riesgo por los restos de comida de barcos internacionales, y en último lugar de vuelos internacionales.

The risk of ASFV introduction into EU by fomites associated to contaminated transports was mainly concentrated in Poland (4.13/5) and Lithuania (3.8/5). This risk was mostly related with the returning trucks from affected areas (65% of the risk by this route), followed by waste from international boats, and finally, from waste from international planes.

IV.

Se desarrolló una herramienta modular que permite la integración y visualización conjunta de los análisis de riesgo realizados para todas las vías de introducción de PPA en la UE. Esta herramienta y los resultados incorporados en ella, podrán emplearse por los países miembros de la UE para la adaptación y actualización de los programas de vigilancia y prevención al actual riesgo existente.

A modular framework was developed for the integration and global visualization of the risk assessments performed for all the introduction pathways of ASFV into the EU. The employment of such tool and the results contained on it by the EU member states would help the adaptation and update of the surveillance and prevention programs to the current risk situation.

V.

Se desarrolló una metodología basada en la combinación de modelos de difusión y modelos estadísticos para la evaluación de los factores de riesgo para PPA presentes en zonas endémicas, tomando como modelo Cerdeña. Estos modelos demostraron la importancia de los cerdos ilegales y las altas densidades de jabalíes en la aparición de brotes de PPA; mientras que la difusión de los mismos está directamente relacionada con medidas de bioseguridad y control de las explotaciones

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A methodology based on the combination of spread and statistic models was developed for the evaluation of risk factors for ASF present in Sardinia. These models demonstrated the importance of illegal pigs and high density of wild boars in the occurrence of ASF outbreaks. Meanwhile the spread of the disease is related with the biosecurity and control measures of the holdings.

VI.

Se desarrolló un estudio retrospectivo en una región de España endémica para PPA con producción de cerdo extensiva, presencia de garrapatas y jabalíes. Los resultados de este estudio demostraron que en esta zona, la población de jabalíes sin otras fuentes de infección secundarias fue incapaz de mantener la infección de vPPA por largos periodos de tiempo.

A retrospective study was developed in one region of Spain endemic for ASF, where extensive production of pigs is present, as well as ticks and wild boar. The results obtained reveal that in that area the population of wild boar was unable to maintain ASFV infection without other sources of infection for a long period of time.

VII.

Se ha detectado por primera vez la presencia de anticuerpos frente al vPPA en el fluido oral de animales infectados. Se adaptaron dos técnicas diagnósticas (ELISA e IPT) disponibles para el diagnóstico serológico de PPA en fluido oral.

This is the first detection of antibodies against ASFV in oral fluid of infected pigs. Two serological techniques for ASF were adapted for the detection of antibodies against ASFV in oral fluid samples.

VIII.

En cuadros de PPA agudos con presencia de elevada fiebre y mortalidad repentina, el fluido oral no sería una buena opción, ya que los animales enfermos pierden el interés por la cuerda y esto imposibilita la obtención de muestra. No obstante, se consideró el fluido oral como una muestra útil para el diagnóstico de PPA en los planes de vigilancia serológica para la detección de animales supervivientes o portadores.

In acute cases of ASF that course with high fever and sudden mortality, oral fluid would not represent a good alternative for diagnosis, as diseased animals have no interest n the rope and sampling is very difficult. However, oral fluid was considered as a very useful approach for the diagnosis of ASF and its potential application in the ASF serological surveillance activities for the detection of survivor and carrier animals.

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9. ANEXOS

9.1. Otras publicaciones científicas

Virus Research 173 (2013) 191–197

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Review

Epidemiology of African swine fever virus

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ABSTRACT

African swine fever virus used to occur primarily in Africa. There had been occasional incursions into Europe or America which apart from the endemic situation on the island of Sardinia always had been successfully controlled. But following an introduction of the virus in 2007, it now has expanded its geographical distribution into Caucasus and Eastern Europe where it has not been controlled, to date. African swine fever affects domestic and wild pig species, and can involve tick vectors. The ability of the virus to survive within a particular ecosystem is defined by the ecology of its wild host populations and the characteristics of livestock production systems, which influence host and vector species densities and interrelationships. African swine fever has high morbidity in naïve pig populations and can result in very high mortality. There is no vaccine or treatment available. Apart from stamping out and movement control, there are no control measures, thereby potentially resulting in extreme losses for producers. Prevention and control of the infection requires good understanding of its epidemiology, so that targeted measures can be instigated.

Roth JA, Richt JA, Morozov IA (eds): Vaccines and Diagnostics for Transboundary Animal Diseases. Dev Biol (Basel). Basel, Karger, 2013, vol 135, pp 159-165.

African Swine Fever Diagnosis Update

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Key words: African swine fever, diagnosis, livestock diseases

Abstract: African swine fever (ASF) is one of the most complex livestock diseases. The significant losses that it causes, coupled with the lack of a vaccine against ASF virus and the possible resemblance with other swine hemorrhagic diseases, make early detection and laboratory diagnosis essential for controlling and managing the disease.

All the techniques currently used to diagnose ASF have been fully validated showing high sensitivity and specificity to detect both antigen and antibodies against all 22 known genotypes; and enable the correct diagnosis of ASF in all possible epidemiological situations. Because no vaccine is available, the presence of antibodies always indicates previous infection, and serological diagnosis must always be performed in parallel with antigen detection to increase the sensitivity and specificity of the analyses. Recent developments in ASF diagnosis, specifically the new field diagnostic tests, have improved and facilitated the likelihood of ASF early detection, essential to fighting the disease.

OPEN ACCESS Freely available online

PLOS ONE

Introduction of African Swine Fever into the European Union through Illegal Importation of Pork and Pork Products

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Abstract

Transboundary animal diseases can have very severe socio-economic impacts when introduced into new regions. The history of disease incursions into the European Union suggests that initial outbreaks were often initiated by illegal importation of meat and derived products. The European Union would benefit from decision-support tools to evaluate the risk of disease introduction caused by illegal imports in order to inform its surveillance strategy. However, due to the difficulty in quantifying illegal movements of animal products, very few studies of this type have been conducted. Using African swine fever as an example, this work presents a novel risk assessment framework for disease introduction into the European Union through illegal importation of meat and products. It uses a semi-quantitative approach based on factors that likely influence the likelihood of release of contaminated smuggled meat and products, and subsequent exposure of the susceptible population. The results suggest that the European Union is at non-negligible risk of African swine fever introduction through illegal importation of pork and products. On a relative risk scale with six categories from negligible to very high, five European Union countries were estimated at high (France, Germany, Italy and United Kingdom) or moderate (Spain) risk of African swine fever release, five countries were at high risk of exposure if African swine fever were released (France, Italy, Poland, Romania and Spain) and ten countries had a moderate exposure risk (Austria, Bulgaria, Germany, Greece, Hungary, Latvia, Lithuania, Portugal, Sweden and United Kingdom). The approach presented here and results obtained for African swine fever provide a basis for the enhancement of risk-based surveillance systems and disease prevention programmes in the European Union.

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Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Assessing the Risk of African Swine Fever Introduction into the European Union by Wild Boar

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Keywords:
African swine fever; risk assessment; European Union; wild boar pathway

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Summary

The presence of African swine fever (ASF) in the Caucasus region and Russian Federation has increased concerns that wild boars may introduce the ASF virus into the European Union (EU). This study describes a semi-quantitative approach for evaluating the risk of ASF introduction into the EU by wild boar movements based on the following risk estimators: the susceptible population of (1) wild boars and (2) domestic pigs in the country of origin; the outbreak density in (3) wild boars and (4) domestic pigs in the countries of origin, the (5) suitable habitat for wild boars along the EU border; and the distance between the EU border and the nearest ASF outbreak in (6) wild boars or (7) domestic pigs. Sensitivity analysis was performed to identify the most influential risk estimators. The highest risk was found to be concentrated in Finland, Romania, Latvia and Poland, and wild boar habitat and outbreak density were the two most important risk estimators. Animal health authorities in at-risk countries should be aware of these risk estimators and should communicate closely with wild boar hunters and pig farmers to rapidly detect and control ASF.

Geospatial Health 8(2), 2014, pp. 489-501

Evaluation of the spatial patterns and risk factors, including backyard pigs, for classical swine fever occurrence in Bulgaria using a Bayesian model

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Abstract. The spatial pattern and epidemiology of backyard pig farming and other low bio-security pig production systems and their role in the occurrence of classical swine fever (CSF) is described and evaluated. A spatial Bayesian model was used to explore the risk factors, including human demographics, socioeconomic and environmental factors. The analyses were performed for Bulgaria, which has a large number of backyard farms (96% of all pig farms in the country are classified as backyard farms), and it is one of the countries for which both backyard pig and farm counts were available. Results reveal that the high-risk areas are typically concentrated in areas with small family farms, high numbers of outgoing pig shipments and low levels of personal consumption (i.e. economically deprived areas). Identification of risk factors and high-risk areas for CSF will allow to targeting risk-based surveillance strategies leading to prevention, control and, ultimately, elimination of the disease in Bulgaria and other countries with similar socio-epidemiological conditions.

Keywords: backyard pig farming, low biosecurity premises, classical swine fever, risk factors, Bayesian modelling, Bulgaria.

En preparación:

- Mur, L., Igolkin, A., Varentsova, A., Pershin, A., Remyga, S., Shevchenko, I., Zhukov, I., Sánchez-Vizcaíno, J.M. Detection of African swine fever antibodies in experimental and field samples from Russian Federation, implications for control. Enviada para su publicació.
- Giménez-Lirola, LG., Mur, L., Rivera, B., Wang, C., Lizano, S., Goodell, C., Rowland, R., Harris, DL., Gallardo, C., Arias, M., Sánchez-Vizcaíno, JM., Zimmerman, J., 2014. Detection of African swine fever virus antibodies in serum or oral fluid specimens using a recombinant protein 30 (rp30) indirect ELISA.
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9.2.Transferencia de resultados

9.2.1. Congresos internacionales

1. Feliziani, F., Mur, L., Iscaro, C., Bandino, E., Liciardi, M., Oggiano, A., Ruiu, A., Rolesu, S., Sánchez Vizcaíno, J.M., 2014. Indagine sulla presenza di *Ornithodoros erraticus* in Sardegna: risultati preliminari. V Workshop Nazionale di Virologia Veterinaria. 26-27 June 2014. Teramo, Italia.
2. Giménez-Lirola, LG., Mur, L., Rivera, B., Wang, C., Lizano, S., Goodell, C., Rowland, R., Harris, DL., Gallardo, C., Arias, M., Sánchez-Vizcaíno, JM., Zimmerman, J. , 2014. The African swine fever rp30 ELISA detects antibody in serum and/or oral fluid specimens. 23nd International Pig Veterinary Society Congress. 9-11 Junio, Cancún, México.
3. Giménez-Lirola, LG., Mur, L., Rivera, B., Wang, C., Rowland, R., Harris, DL., Gallardo, C., Arias, M., Zimmerman, J., Sánchez-Vizcaíno, JM, 2014. Multiplex Luminex® assay for detection of antibodies against three major proteins of ASFV. 23nd International Pig Veterinary Society Congress. 9-11 Junio, Cancún, México.
4. Giménez-Lirola, LG., Mur, L., Mogler, M.A., Lizano, S., Goodell,C.K., Sanchez-Vizcaino, JM., Harris, DLH., Zimmerman, J., 2014. RNA particles: A novel application for efficient development of FAD serologic assays. AASV 45th annual meeting. March 1-4, 2014, Dallas, Texas, USA.
5. Mur, L., Martínez-López, B., Feliziani, F., Sánchez, A., Sánchez-Vizcaíno, JM., 2013. Expert opinion on controlling African swine fever in Sardinia. 2013 Annual Conference of the Society for Veterinary Epidemiology And Preventive Medicine. 20-22 Marzo, 2013. Madrid, España.
6. Sánchez Rodríguez, A., Mur, L., A., Martínez-López, B., Sánchez-Vizcaíno, JM., 2013. Survey-based study on African swine fever in 13 African countries. 2013 Annual Conference of the Society for Veterinary Epidemiology And Preventive Medicine. 20-22 Marzo, 2013. Madrid, España.
7. Sánchez-Vizcaíno, JM., Mur, L., Martinez-Lopez, B., 2012. African swine fever (ASF): five years around Europe. Plenary lecture. IX International Congress of Veterinary Virology and Joint meeting with the European Society of Clinical Virology. Madrid, Spain. 4-7 Septiembre, 2012.
8. Mur, L., Gallardo, C., Soler, S., Zimmermman, J., Pelayo, V., Sanchez-Vizcaino, JM., Arias, M., 2012. Potential use of oral fluid samples for African swine fever antibody detection. IX International Congress of Veterinary Virology and joint meeting with the European Society of Clinical Virology. 4-7 Septiembre, 2012. Madrid, España (1er premio ESVV Isabel Mínguez Tudela a la mejor presentación oral de jóvenes científicos).
9. Mur, L., Martinez-Lopez, B., Costard, S., de la Torre, A., Jones, BA.. Pfeiffer, D., Sánchez-Vizcaíno, JM., Wieland, B., 2012. General framework for the risk assessment of African swine fever introduction into the European Union. IX INTERNATIONAL CONGRESS OF VETERINARY VIROLOGY and joint meeting with the European Society of Clinical Virology. 4-7 Septiembre, 2012. Madrid, España.

10. Gallardo, C., Soler, A., Nieto, R., Mur, L., Perez, C., Pelayo, V., Martins, C., Sánchez-Vizcaíno, JM., Arias, M., 2012. Protection of European domestic pigs from Armenia virulent African swine fever virus by experimental immunisation using the attenuated and non-haemadsorbing African swine fever virus isolate ASFV/NH/P68. IX International Congress of Veterinary Virology and joint meeting with the European Society of Clinical Virology. 4-7 Septiembre, 2012. Madrid, España.

11. Costard, S., Jones, B.A., Martinez-Lopez, B., Mur, L., De La Torre, A., Martinez, M., Sanchez-Vizcaino, F. Sanchez-Vizcaino, J.M., Pfeiffer, D.U. and Wieland, B. 2012. Risk of African swine fever introduction into the European Union through illegal importation of pork and products. Oral communication. 13th International Symposium on Veterinary Epidemiology and Economics. 20-24 Agosto, 2012. Maastrich, Holanda.

12. Mur, L., Martínez-López, B. and Sánchez-Vizcaíno, J.M., 2012. Transport-associated fomites: estimation of the potential risk for African swine fever introduction into European Union". Poster. 13th International Symposium on Veterinary Epidemiology and Economics. Maastrich, Holanda. 20-24 Agosto, 2012.

13. Martínez-López, B., Mur, L., Rodríguez-Prieto, V., Sánchez-Vizcaíno, F. and Sánchez-Vizcaíno, J.M., 2012. Estimation of back yard pig (BYP) density in the EU: first step to evaluate the role of BYP in disease transmission". Poster. 13th International Symposium on Veterinary Epidemiology and Economics. Maastrich, Holanda. 20-24 Agosto, 2012.

14. Gallardo, C., Nieto, R., Mur, L., Soler, A., Pelayo, V., Bishop, R., Sánchez-Cordón, P. Martins, C., Sánchez-Vizcaíno, JM., Arias, M., 2012. African swine fever (ASF) in Africa. The role of the African indigenous pigs in the transmission of the disease. Plenary Lecture. 6th Annual Meeting EPIZONE, 12 - 14 Junio, 2012. Brighton, United Kingdom.

15. De la Torre, A., Bosch, J., Iglesias, I., Martínez, M., Mur, L., Martínez-López, B., Sánchez-Vizcaíno, JM., Muñoz, MJ., 2012. Risk of African swine fever introduction into the European Union (EU) through wild boar pathway. 6th Annual Meeting EPIZONE, 12 - 14 Junio, 2012. Brighton, United Kingdom.

16. Iscaro, C., Giammarioli, M., Duarte, M., Fernandez-Pinero, J., Gunter K.M., Health, L.E., Leblanc, N., McKillen, J., Mur, L., Oggiano, A., Sanz, A., Stahl, K., Severi, G., De Mia, G.M., 2012. Ring trial on ASFV novel molecular diagnostic techniques. Poster. 6th Annual Meeting EPIZONE, 12 - 14 Junio, 2012. Brighton, United Kingdom.

17. Sánchez-Vizcaíno, JM., Mur, L., Martínez-López, B., 2011. African Swine Fever update. Plenary lectures. International Symposium on Emerging and Re-emerging Pig Diseases. 12-15 Junio. Barcelona, España.

18. Wieland, B., Costard, S., Martinez-Lopez, B., Mur, L., Martinez, M., Jones, B., Sanchez-Vizcaino, F., Sanchez-Vizcaino, JM., Pfeiffer, D., 2011. Proxy driven approach to assess risk of African Swine Fever introduction into EU countries via illegal imports. Annual Conference of the Society for Veterinary Epidemiology and Preventive Medicine. 23 - 25 Marzo, 2011. Leipzig, Germany.

19. Mur, L., Martínez-López, B., Martínez-Avilés, M., Sánchez-Vizcaíno, JM., 2010. African Swine Fever: a high risk again. 21st International Pig Veterinary Society (IPVS) Congress. 18-21 Julio. Vancouver, Canadá.

9.2.2. Otras publicaciones

1. Sánchez-Vizcaíno, JM., Mur, L., Sánchez-Matamoros, A., Martínez-López, B., 2014. Peste porcina africana: nuevos retos y medidas para evitar su propagación. 82ª Sesión General OIE. Asamblea Mundial. París 25-30 mayo, 2014.
2. Mur, L. y Sánchez-Vizcaíno, JM., 2014. Gestión de crisis sanitarias en ganado porcino. Curso de formación continuada. SUIS, vol. 106, 107, 108.
3. Sanchez-Vizcaino, JM., Mur L., 2012. African swine fever: a reemerging risk from East Europe. OIE Bulletin 2012-4.
4. Zaberezhnyĭ AD, Aliper TI, Grebennikova TA, Verkhovskiĭ OA, Sanchez-Vizcaino JM, Mur L, Nepoklonov EA, L'vov DK., 2012. African swine fever in Russian Federation. Vopr Virusol.. 2012: 57(5):4-10
5. Mur L y Sánchez-Vizcaíno JM., 2012. Peste porcina africana. Reconocer la enfermedad en campo.3tres3.com. 2012/3/30
6. Sánchez-Vizcaíno JM. y Mur L. La PPA: Breve visión desde el pasado, presente y... futuro?. 3tres3.com. 2012/1/24.
7. Mur, L., Martínez-López, B., Sánchez-Vizcaíno, JM., 2011. African swine fever: are we aware? Pig progress. The international magazine on pig production. Vol.27. pp7-
8. Mur, L., Martínez-López, B., Martínez-Avilés, M., Sánchez-Vizcaíno, JM.,2010. African swine fever: Import of live pigs not a high risk. Pig progress. The international magazine on pig production. IPVS Focus 2010.
9. Martínez, M., Martínez-López, B., Muñoz, M.J., de la Torre, A., Mur, L., Sánchez-Vizcaíno, JM., 2009. Expansión de peste porcina africana en el mundo: Riesgo de entrada en España. Gaceta Sanitaria. Vol.23 Especial Congreso 3, 28.
10. Mur, L., Martínez-López, B., Sánchez-Vizcaíno, JM., 2009. El despertar de la peste porcina africana. Revista Complutense de Ciencias Veterinaria. Vol. 3 (2). 149-158.
11. Mur, L., Martínez-López, B., Sánchez-Vizcaíno, JM., 2009.Virus Ebola-Reston. ¿El cerdo, un nuevo hospedador? Revista Complutense de Ciencias Veterinarias. 3 (2) 159-166.
12. Mur, L. y Sánchez-Vizcaíno, JM., 2008. La vida en un laboratorio de alta seguridad biológica. Revista Complutense de Ciencias Veterinaria. Revista Complutense de Ciencias Veterinaria. Vol. 2 (2).160-165. 2008. ISSN:1988-2668

13. **Espacio web:** Emergentes online-Peste porcina Africana, donde se encuentra información sobre la epidemiología de la PPA y todas las novedades referentes a esta enfermedad (http://www.sanidadanimal.info/sanidadanimal/es/actividades/emergentes-online/peste-porcina-africana-ppa.html)
14. **Curso online sobre PPA**, claves para su prevención y control. Trabajo de divulgación, dentro del proyecto ASFORCE. (http://asforce.org/course/#)
15. **CD interactivo**. African swine fever diagnosis. Sánchez- Vizcaíno, JM., Mur, L., Arias, M. ISBN 978-84-693-6137-5. Traducido a cinco idiomas: inglés, ruso, chino, francés y español.

9.2.3. Cursos internacionales

1. Training course on African swine fever (ASF) diagnosis. Central Veterinary laboratory-Kabete, Nairobi, Kenya. January 2014.
2. Training course on epidemiology and diagnosis of African swine fever (ASF), Classical swine fever (CSF) and other swine diseases of high impact. IZS dell'Umbria e Marche. Perugia, Italy. November 2013.
3. Training workshop for African swine fever (Bulgaria). Sofia, Bulgaria. September 2013.
4. Training workshop for African swine fever (Spain). Universidad Complutense de Madrid. April 2013
5. Regional Training Course on Major Transboundary and Zoonotic Animal Diseases: Early detection, Surveillance and Epidemiology-Focus on African Swine Fever, Capripox and Rabies. Laboratoire National Veterinaire. Garoua, Cameroon. July 2012
6. Regional Training Course on early and rapid nuclear and nuclear-related diagnostic and tracing technologies for African and Classic Swine Fever. IAEA, Austria. May, 2012.
7. Training course on African swine fever diagnosis. Universidad Complutense de Madrid. December, 2011.
8. International training course on African swine fever (ASF) Epidemiology and Laboratory Diagnosis (ASF EPI-LAB). Universidad Complutense de Madrid. July, 2011.
9. Training course on African swine fever (ASF) Epidemiology. LANADA, Bingerville, Côte d´Ivoire. November, 2010.
10. Training course on African swine fever (ASF) Epidemiology and Diagnosis. Lanzhou Veterinary Research Institute, China. September, 2010.